THE ASSOCIATION BETWEEN NON-ESTERIFIED FATTY ACIDS AND Beta-HYDROXYBUTYRATE DURING THE TRANSITION PERIOD IN HOLSTEIN DAIRY COWS AND NEGATIVE DOWNSTREAM OUTCOMES – DISEASE INCIDENCE, MILK PRODUCTION AND REPRODUCTION

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THE ASSOCIATION BETWEEN NON-ESTERIFIED FATTY ACIDS AND β-HYDROXYBUTYRATE DURING THE TRANSITION PERIOD IN HOLSTEIN DAIRY COWS AND NEGATIVE DOWNSTREAM OUTCOMES – DISEASE INCIDENCE, MILK PRODUCTION AND REPRODUCTION

Paula Andrea Ospina, Ph. D.

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The 3 weeks pre-partum to 3 weeks post-partum is a challenging time for dairy cows because of both environmental and biological changes taking place in preparation for parturition and lactation. At the animal level, excessive negative energy balance (NEB) can increase the risk of developing displaced abomasa, clinical ketosis, metritis, and/or retained placenta and can negatively affect reproduction and milk production. Non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA) are measurable blood metabolites used to estimate the level of NEB. The objectives of herein were to: 1) identify critical concentrations of NEFA and BHBA above which the development of diseases were more likely; 2) describe statistical methods for best evaluating these effects as risk ratios; 3) identify the NEFA and BHBA critical concentrations associated with decreased reproductive performance and milk production; 4) evaluate the herd alarm level, i.e., the association between the proportion of animals sampled that had NEFA and BHBA concentrations above the thresholds and herd-level disease incidence, reproductive, and milk production outcomes; 5) compare the results of pooling samples versus evaluating individual animals that were above a herd alarm level; and 6) evaluate the herd-level sensitivity, specificity, and herd predictive value positive and negative when using individual samples to estimate herd-level risk. Animals with elevated concentrations of pre- and post-partum NEFA

(0.3 and 0.7 mEq/L, respectively) and post-partum BHBA (12 mg/dL) were more likely to develop diseases; less likely to get pregnant. The effects of elevated metabolites on milk production at the individual animal level were different between cows and heifers, cows produced less milk while heifers produced more. However, when animals were evaluated at the herd level (i.e., when more than 15-20% of the animals sampled had metabolite concentrations above the critical thresholds) elevated NEFA and BHBA were associated with decreased milk in all groups. When evaluating NEB at the herd-level, pooled samples are not recommended because they have low sensitivity. It is recommended to sample 15 animals to maximize HPV- and 20 to maximize HPV+. The information acquired from this research will help improve transition cow monitoring and management strategies.

BIOGRAPHICAL SKETCH

Paula Andrea Ospina was born on May 28th, 1978 in Cali, Colombia. Paula is the eldest of three daughters and she was 7 years old when her family immigrated to Los Angeles, California. Paula had to overcome several challenges, including but not limited to: learning the English language, adapting to a new culture, living in very modest conditions, and taking care of her younger sisters while her mother, Blanca, worked and went to school full-time.

Although Blanca worked very hard, it was difficult to support three children on a very modest salary and for about 1 year (1988-1989) Paula's family moved through homeless shelters while Blanca learned English and was able to improve her earning wages. Unfortunately, Paula's father was unwilling to complete the journey which he started with his family, and he returned to their native country in the early 90's. At this time, Blanca was mid-way through her college education and would go onto complete her Bachelors of Science degree in Computer Science. After graduation, she began working as a teacher in the Lynwood Unified School District (a suburb of Los Angeles), and Paula's family was able to move to a better neighborhood.

Although Paula did not consider herself truly bilingual until about the ninth grade, she did well in the educational system and due to her high achievements she was accepted into the California Academy of Math and Science (CAMS). This high school is located on the California State University Dominguez Hills Campus and its mission is to increase the nation's pool of graduates in mathematics and science. When Paula was accepted into this program, she was attending junior high school at Whaley Middle School in Compton, California.

When Paula started high school, she felt like she had finally found her niche. She was able to attend and learn while in class! She was no longer distracted by all of the discipline

problems that plague some of the schools she had attended. She was also able to take college classes for college credit and participated in varsity sports like cross-country and track. She became captain of the cross-country team as a junior and did well in competitions. Paula focused on taking math and science courses while in high school because she knew that going to veterinary school was going to be a challenge that would require a lot of math and science. She attended California Polytechnic State University, Pomona and received an undergraduate degree in Animal Science focusing on the pre-vet option in May of 1999 and began her veterinary degree at the Cornell University College of veterinary medicine. In May 2003, Paula completed her veterinary degree from one of the most prestigious universities in the United States.

After graduation, Paula returned home, southern California, to spend time with her family. She worked at a small animal private practice and gained valuable clinical experience. Although she had learned a great deal from private practice, she felt that she would make a better impact on the health and lives of animals at a different level. She completed a Master's degree in public health from the University of California at Davis on May of 2007 and returned to Cornell University to a Doctoral program in the field on animal science focusing on epidemiology. Her research focus has been transition cow energy metabolism. While working on her PhD, she applied for a position in the animal science department at Cornell. She was excited to accept the position as a senior lecturer and since August 2009 she has been working on her PhD through the employee degree program. As a senior lecturer most of her time is dedicated to teaching, research, and extension. She enjoys her interaction with the students because they are very involved and receptive to learning and she is gradually expanding on her research and extension role. Paula's career objectives are to continue working with students and continue to develop her research and extension roles.

Dedicated to Whogo (a.k.a fat cat) & Itchy

~Who stayed up with me… even if it was passed their bed time~

Muchos Besos

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I would like to thank everyone that helped me through this journey, but I would like to start with my family: mama Blanca, Beatriz, and Ruby. Mama Blanca you helped me navigate through some of the hardest classes I have ever taken. Gracias. Beatriz and Ruby, you were there for me when I needed to talk and you listened. Thanks. Brian, who has become a new part of my family but whose company, support, hand holding (literally) and *tomfoolery* I could not do without. Besos.

I have to give a special thanks to Dr. Daryl Nydam, because without him, I would not be at Cornell. He personally helped me finish my application and walked it over to the Animal Science Department. Daryl has also been and will continue to be a great mentor and advisor. I would also like give a special thanks to the other members of my committee: Dr. Tom Overton- for being supportive and giving me a job; Dr. Charles Guard - for letting me ride along and continue learning about veterinary medicine; and Dr. Tracy Stokol – for having my back and letting me know that if the *guys* were not letting me do what I needed to do that she would be my advocate. I would also like to thank my friends, colleagues, and classmates: Dr. Geoffrey Groocock, Dr. Jessica McArt and Dr. Jennifer Zambriski, thanks for all the support and help.

Obviously most of this could not have happened without the help of Charlene Ryan helping to collect the samples, Richard DeFrancisco for helping me run the samples, and Brooke Crosier for helping put the all of this data together. The support from the farms that let us collect information cannot be overlooked; it is through their support that we were able to bring this information together. I would like to acknowledge the USDA federal formula funds and USDA NRI for financial support of this research project. Lastly, I would like to acknowledge the

personal financial support provided by the Cornell University diversity fellowship program which helped fund the first two years of my PhD.

Thanks and I hope that this is just the beginning of future collaborations.

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LIST OF ABBREVIATIONS

AcAc	Acetoacetate
BCS	Body condition score
BHBA	Beta-hydroxybutyrate
bST	Bovine somatotropin
CK	Clinical ketosis
CPT-1	Carnitine palmitoyltransferase
DA	Displaced abomasum
DMI	Dry matter intake
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPV-	Herd level predictive value negative
HPV+	Herd level predictive value positive
HSe	Herd level sensitivity
HSp	Herd level specificity
LR-	Likelihood ratio negative
LR+	Likelihood ratio positive
MCM	Methylmalonyl-CoA mutase
ME 305	Mature Equivalent 305 day milk yield
MET	Metritis
NAHMS	National Animal Health Monitoring System
NEB	Negative energy balance
NEFA	Nonesterified fatty acid

OAA	Oxaloacetate
OLS	Ordinary least squares
OR	Odds ratio
PC	Pyruvate carboxylase
PCoAc	Propionyl-CoA carboxylase
PEP	Phosphoenolpyruvate
PEPCK	Phophoenolpyruvate carboxykinase
PG	Propylene glycol
PPAR-gamma	Peroxisome proliferator-activated receptor-gamma
PR	Pregnancy rate
ROC	Receiver operator characteristic
RP	Retained placenta
RPC	Rumen protected choline
RR	Risk ratio
SCK	Sub clinical ketosis
TAG	Triglycerides
TCA	Tricarboxylic acid cycle
TMR	Total mixed ration
TP	True prevalence
TZD	Thiazolidinedione
VLDL	Very low density lipoproteins
VWP	Voluntary waiting period

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The following chapters of this dissertation will discuss: the objective thresholds of markers of excessive negative energy balance (**NEB**) during the transition period, and their association with negative downstream outcomes at both the individual animal and herd-level; a method to estimate risk ratios to evaluate this association when applicable; and the consequences of varying test sensitivity and specificity on herd-level parameters such as herd predictive value positive and negative. The outcomes of interest were disease development (displaced abomasa, clinical ketosis, metritis and/or retained placenta); mature milk equivalent 305 (**ME 305**) based on 4 test days; and reproduction. The metabolites, non-esterified fatty acids (**NEFA**) and beta-hydroxybutyrate (**BHBA**) have been associated with NEB and the relative ease of measurement has made them useful for evaluation of NEB during the transition period. The following literature review will describe the physiological background and contemporary information which was the basis for the development of the current study objectives.

Define the transition period

In response to lactogenesis, within a few days post-partum the demands for glucose, amino acids, and fatty acids are several fold higher (2.7, 2.0, and 4.5 times respectively) what they are pre-partum (Bell, 1995). Although the concept of homeorhesis (Bauman and Currie, 1980) has been a good foundation for understanding the coordination of the events necessary to meet the energy demands early in lactation, these events are complex and dynamic. To complicate matters, a decrease in dry matter intake (**DMI**) starting pre-partum (Hayirli et al.,

2002) limits the availability of the substrates, however it is important to note that even when DMI is forcibly maintained in the pre-partum period, some of the energy management mechanisms still take effect, i.e., fat is still mobilized thus NEFA is still elevated around parturition (Bertics et al., 1992).

The time period in which these mechanisms unfold is routinely referred to as the transition period, which is generally confined to 3 weeks pre-partum to 3 weeks post-partum (Grummer, 1995; Drackley, 1999). Although research on the changes that take place during the transition period and their association to downstream outcomes can be traced back to 1980s (Herdt et al., 1981b), the association between energy, the transition period and negative downstream outcomes was still difficult to evaluate. For example, Herdt et al., (1981) recommended sampling BHBA in at least 7 animals to identify whether a herd was at increased risk of developing subclinical ketosis (**SCK**); however, this research group also reported that neither BHB nor glucose could be used as valid indicators of energy balance (Herdt et al., 1981a).

It was not until the 1990s that significant progress on this topic was made at both the nutritional and metabolic level (Bell, 1995; Grant and Albright, 1995; Grummer, 1995; Goff and Horst, 1997; Drackley, 1999). This lag in research of the transition period may be due to the fact that this period is a very dynamic time period for a cow, most diseases are likely to occur early in lactation (Ingvartsen et al., 2003), and thus the potential variability between animals and herds has been difficult to study (Drackley, 1999). To illustrate the complexity in interpreting information from research during this time period, take for example the contradiction in results between studies that demonstrated that high energy diets (from fat or other sources) in the dry

period were beneficial (McNamara et al., 2003; Douglas et al., 2004) versus more current research which contradicts these findings (Douglas et al., 2006; Janovick et al., 2011).

Although this review will not focus on the details of management, environmental considerations, nutrition, or genetics these are all important factors which can affect the transition period and can be associated with a successful lactation. Dairy cows under current management conditions routinely undergo several pen moves during the transition period and these non-dietary factors can negatively affect performance and health during the transition period (Contreras and Overton 2004). More recent research has looked at stress, measuring cortisol effects in the blood and feces, and inflammation, measuring acute phase proteins such as haptoglobin (Huzzey et al., 2011) as risk factors of the development of negative downstream outcomes. Although Huzzey et al., (2011) reported that NEFA was a better predictor of the odds of developing disease, cows still experience stress and inflammation during the transition period (Bossaert et al., 2011) and stress can increase NEFA concentrations (Leroy et al., 2011).

Although this is a very brief description of factors to consider during the transition period, they play a very important role especially as cow rations are better formulated to meet the energy needs of dairy cows. In fact, recent research which evaluated milk production in 47 herds receiving the same diet suggests that over 50% of the observed variation in milk yield across dairy farms is not related to nutrition but rather to management or environmental factors (Bach et al., 2008).

In addition to the environment, genetic factors may also play a role in a cow's ability to transition well. Research specifically evaluating the heritability of the levels of NEFA and BHBA in transition dairy cows has been reviewed in a herd in Greece (Oikonomou et al., 2008). Although this research suggests that there may be some heritability in the levels of NEFA and

BHBA produced by the cow, this research was performed on only one herd. In order to improve the external validity of this study, the same genetic model was used to evaluate 100 herds in the northeast (USA) and found no significant heritability associated with NEFA and BHBA levels (Ospina et al., not published). However, in these 100 herds, the ratio of sire to daughters was low (889 sires with 2728 daughters) and this low ratio may have interfered with the ability to find the genetic association if present.

Lastly, it is important to note that although the transition period has been generally confined to 3 weeks pre- and post-partum, the entire dry period should be considered a period of transition because it can affect the cow's ability to go through the transition period sometimes even more so than the events that take place directly before parturition. For example, when evaluating energy in diets during the far-off (> 25 d pre-partum) versus close-up (< 25 d pre-partum) period, Dann et al., (2006) reported that overfeeding during the far-off period had a greater negative impact early in the transition period due to DMI, and consequently increased levels of NEFA and BHBA.

Negative energy balance

According to the National Animal Health Monitoring System (**NAHMS**), in the 1940s there were about 25 million cows (USDA, 2007) producing about 2,000 kilograms of milk per year (Capper et al., 2009) and approximately 130 million people (U.S. Census Bureau, 2012a). Currently there are about 9 million cows providing approximately 9,000 kilograms of milk per year and feeding approximately 300 million people (U.S. Census Bureau, 2012a; U.S. Census Bureau, 2012b). Although this improvement in the efficiency of milk production is based on several factors such as management, nutrition, and genetic selection, the cow's ability to achieve this production is innate and arguably based on the concept of homeorhesis, i.e., the cow's ability

to coordinate the metabolism between several body tissues in order to partition nutrients to support the dominant physiological process like pregnancy or lactation (Bauman and Currie, 1980).

Early in lactation, the demand for glucose to support lactogenesis cannot be fulfilled by dietary intake; therefore, dairy cows will enter a state of NEB (Bell, 1995; Grummer, 1995; Overton et al., 1998; Ingvartsen and Andersen, 2000). Fortunately, there are several energy management mechanisms in place at parturition which help to enhance gluconeogenesis from non-carbohydrate precursors and spare glucose utilization (Bell, 1995) to provide the cow the ability to meet the energy demands of lactogenesis. In addition, ketones and NEFA can be utilized for milk fat synthesis (Palmquist et al., 1969). Therefore, with these mechanisms in place, it is normal for a cow to have some level of circulating NEFA and BHBA early in lactation; however, there is a critical point above which detrimental outcomes are more likely and signify an excess of negative energy. The following is a general review of the energy mechanisms in place to help meet the demands of lactogenesis through gluconeogenesis, lipolysis, and ketogenesis and explain how NEFA and BHBA can be used as markers of NEB.

Metabolic hormones in the transition period

Increased concentrations of NEFA and BHBA in the periparturient period are in part due to hormone-activated cues in place to help the cow meet the energy demands of lactogenesis. The following is a general review of the hormones involved (e.g., glucagon, insulin, catecholamines, glucorticoids, estradiol, leptin, and somatotropin) during the periparturient period and their effect on energy mechanisms.

When a cow enters NEB, glucagon is released by the pancreas and promotes gluconeogenesis in the liver by increasing extraction of amino acids from the blood for

conversion to glucose (Brockman et al., 1975). Glucagon infusions have been shown to promote liver glycogenolysis, enhance gluconeogenesis, while not increasing lipolysis from adipose tissue in early lactation (She et al., 1999).

Insulin, which is also released by the pancreas, promotes glucose and ketone utilization, lipogenesis, and opposes lipolysis in insulin sensitive tissues like adipose tissue and skeletal muscle. In the liver, insulin decreases beta-oxidation capacity (Jesse et al., 1986) and increases triglyceride synthesis (Cadorniga-Valino et al., 1997). During the transition period, a cow undergoes a state of insulin resistance (Bell, 1995). Insulin resistance allows the body to react differently even when insulin is elevated, thus lipolysis persists even when insulin is elevated (Vernon and Taylor, 1988) which occurs when there is increased glucose concentration in circulation.

Although the exact mechanism of insulin resistance in adipose tissue is still under investigation, Lemor et al., (2009) reported that there was decreasing adiponectin sensitivity in adipose tissue after calving and this may contribute to insulin resistance by adipose tissue. Sundvold et al., (1997) reported that insulin resistance is likely mediated through the actions of peroxisome proliferator-activated receptor-gamma (**PPAR-gamma**) on adipose tissues. The activation of PPAR-gamma, enhances insulin action and decreases the release of fatty acids from adipose tissue (Guo and Tabrizchi, 2006). Thiazolindinediones (**TZD**) are the most potent ligands of PPAR-gamma (Houseknecht et al., 2002) and given that it enhances insulin action, it may be utilized to reduce lipolysis. Smith et al., (2009) evaluated the effect of 2,4-thiazolidinedione (**TZD**) a potent synthetic ligand for PPAR-gamma and found that TZD administration pre-partum improved metabolic health and DMI in periparturient cows and may decrease dependency on body fat reserves. While studying the effects of high versus low energy

diets in the dry period and the association with TZD on insulin responses, Schoenberg et al. (2011), found that although there was no change in insulin concentrations based on TZD treated, cows treated with TZD tended to be more sensitive to insulin based on an insulin challenge. One of the challenges associated with TZD is that it must be administered via injection and this is impractical in most dairy settings, however, the mechanisms by which this drug works may help develop the understanding of insulin resistance and may lead to better management or other treatment options.

During the transition period, cows can undergo a lot of stress due to diet changes and pen moves. There are several physiological adaptations to stress. Within minutes after a stressful event, there will a rise in catecholamines (e.g., epinephrine and norepinephrine) produced by the adrenal gland. This will increase cardiovascular output, activate the immune system, and increase mobilization of energy reserves, i.e., increase NEFA levels (Sapolsky et al., 2000). In the periparturient dairy cow, there are substantial increases in number of beta-receptors on bovine adipocytes during early lactation compared to a dry cow which makes the peri-parturient cow more responsive to the effects of catecholamines (Jaster and Wegner, 1981). This is important because the enhanced response to stress hormones early in lactation can exacerbate the NEB status of the animal by increasing the amount of circulating NEFA concentrations.

Glucocorticoid (e.g., cortisol) concentrations will also increase in response to stress. In the weeks leading to parturition, cortisol concentrations increase, however, approximately 2 days prior to parturition, the cortisol concentrations increase exponentially (Patel et al., 1996). Initially, the effect of cortisol is to accentuate the actions of catecholamines (Reynaert et al., 1976), however the long term effects of elevated levels of glucocorticoids can decrease over all health due to immunosuppression (Munck et al., 1984), reduced feed intake (Tempel and

Leibowitz, 1994), and compromised reproductive performance (Johnson et al., 1992). The secretion of cortisol is episodic and subject to individual variation (Thun et al., 1981). Also sampled collection may increase levels, making monitoring cortisol difficult. Huzzey et al., (2011), explored the association between fecal and blood cortisol, along with other hormonal and energy-related predictors, and disease. Although this study concluded that NEFA, a marker of NEB, was a better predictor of disease outcomes than cortisol, increased cortisol levels can have a negative impact on reproduction and milk production.

Estradiol concentrations rise throughout pregnancy and peak 1-2 weeks before term (Bell, 1995), this is an important event because it has been implicated in the inappetance of ruminants during late pregnancy (Sechen et al., 1988). However, the direct effects of estradiol on specific tissues have not been established (Grummer et al., 1990), and more recent research has demonstrated that estradiol treatment is not associated with lipolysis or hepatic fatty acid metabolism (Bremmer et al., 1999).

Leptin is synthesized by white adipose tissue and is negatively regulated by undernutrition, i.e., to signal the need for food consumption (e.g. increase appetite) leptin levels are low, and to signal satiety leptin levels rise. Systemic administration of leptin is associated with reduced feed intake and increased energy expidentrue (Ahima and Flier, 2000). During the transition period, at a time when there is a decrease in nutrients, decrease in insulin and an increase in growth hormone concentration, there is also a decrease in leptin concentration (Block et al., 2001; Block et al., 2003). Although the leptin control mechanisms are still under investigation, Thorn et al., (2008) demonstrated that hypoinsulinemia is partly responsible for the decrease of leptin concentrations, and that hyperinsulinemia was associated with an increase in leptin concentration.

Early in the post-partum period, plasma concentrations of somatotropin rise and have a natural increase at parturition and this is the primary homeorhetic regulator during the transition from pregnancy to lactation (Bell, 1995). By opposing tissue response to insulin, somatropin also decreases rates of lipogenesis (Bauman and Vernon, 1993) and helps partition glucose for mammary use. Additionally, differences in growth hormone receptors in the liver compared to skeletal muscle suggests a role of somatotropin in regulating tissue-specific changes in responsiveness during the transition period (Wook Kim et al., 2004).

Gluconeogenesis in the transition period

Glucose management in ruminants is of particular importance because ingested carbohydrates are routinely fermented to short-chain fatty acids by rumen microbes; therefore glucose must be synthesized by the liver (Reynolds et al., 1988). The three most abundant fatty acids are: propionate, butyrate, and acetate; however of these three, only propionate can be used as a precursor for the synthesis of glucose with valerate and isobutyrate also available, but used significantly less (Bergman, 1990; Reynolds et al., 2003). It is important to note that insulin does not inhibit hepatic gluconeogenesis from propionate (Donkin and Armentano, 1995) and excessive amounts of propionate can lead to propionic aciduria which can have negative consequences on health (Allen et al., 2005; Deodato et al., 2006). Rather, hepatic gluconeogenesis from propionate is regulated through the supply of glucose precursors, enzyme activity, and end-product feedback. The ability to control the supply of glucose precursors will be discussed in a later section, under the use of ionophores.

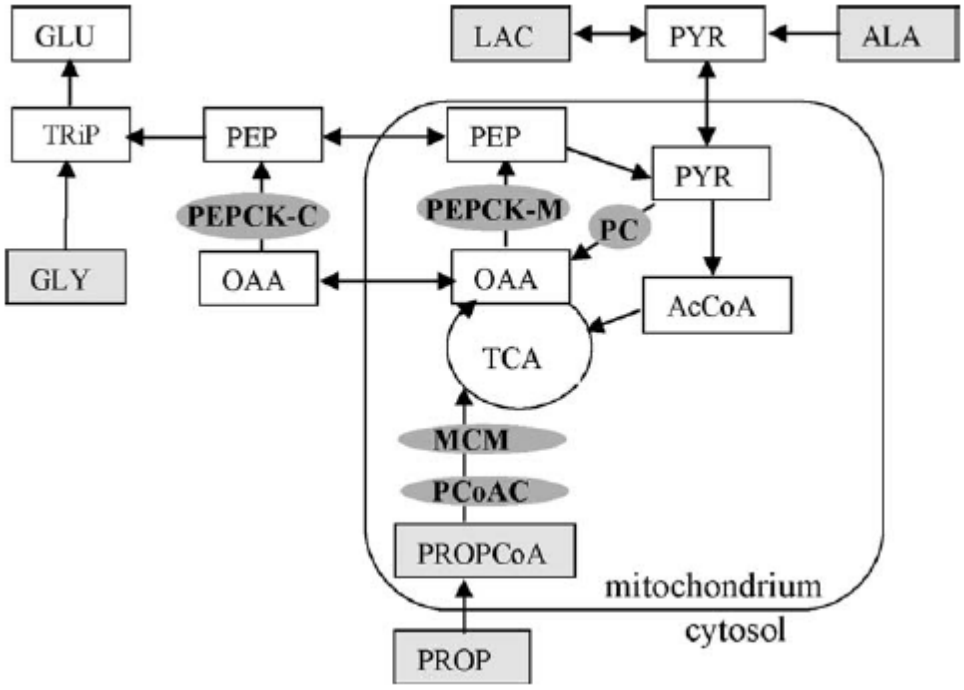
The factors associated with propionate gluconeogenesis will be discussed and an illustration of the control points for glucogenic precursor entry into gluconeogenesis the bovine liver is found in Figure 1. A review by Aschenbach et al., (2010) reported that propionate is

converted through mitochondrial propionyl-CoA carboxylase (**PCoAC**), methylmalonyl-CoA mutase (**MCM**), and part of the tricarboxylic acid (**TCA**) cycle to oxaloacetate (**OAA**). Once OAA has been created it is metabolized by phophoenolpyruvate carboxykinase (**PEPCK**) to phosphoenolpyruvate (**PEP**), and then to glucose or it can serve as an acetyl-CoA acceptor in the TCA cycle. Because PCoAC, MCM, and PEPCK-M are necessary for propionate entry into gluconeogenesis, it seems likely that these may be control points in gluconeogenesis from propionate. Although not much is currently known about the transcriptional or translational regulation of MCM and PCoAC in bovines, the enzyme MCM is dependent on vitamin B12 and peripartal administration of vitamin B12 can have positive effects on the metabolic status of high yielding dairy cows (Rollin et al., 2010). Additionally, increasing propionate availability by feeding ionophores pre-partum induces hepatic PEPCK-C mRNA expression. It is also important to note that pyruvate carboxylase (**PC**), an enzyme used for amino acid entry into gluconeogenesis, is elevated during feed restriction while PEPCK is elevated with bovine somatotropin (**bST**) treatment (Velez and Donkin, 2004; Velez and Donkin, 2005). This is an important adaptation, because pyruvate (created with PC) can be used to maintain OAA availability to support hepatic gluconeogenesis.

After parturition, there is a decrease in insulin and less is stimulated from the pancreas (Drackley et al., 2001). A decrease in insulin concentration results in glucose sparing, due to decreased glucose utilization by insulin sensitive organs, and this allows the mammary gland to have additional access to glucose for lactogenesis (Komatsu et al., 2005). Gluconeogenesis is closely linked to lactogenesis because the amount of glucose available for lactogenesis will determine the amount of milk produced (Mepham, 1993). In addition to dictating milk production, glucose is a fundamental nutrient required by the brain and other tissues to function

and changes are not tolerated without adverse effects on the health of the animal, therefore it is under tight homeostatic control and concentrations will usually remain within normal physiologic levels.

Figure 1.1. Control points for glucogenic precursor entry into gluconeogenesis in bovine liver. The relative entry rates of propionate, lactate, and alanine can be regulated via differential expression of pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCoAC), and the cytosolic and mitochondrial isoforms of phosphoenolpyruvate carboxykinase (PEPCK-C and PEPCK-M). Abbreviations: AcCoA, acetyl CoA; ALA, alanine; GLU, glucose; GLY, glycerol; LAC, lactate; OAA, oxalaoacetate; PEP, phosphoenolpyruvate; PROP, propionate; PROPCoA, propionyl CoA; PYR, pyruvate; TCA, tricarboxylic acid cycle; TRiP, triose phosphates . (Recreated from Figure 2 in Aschenbach et al., 2010)



Lipolysis

NEFA and NEB

The excess energy stored in adipose tissue can be used during times of NEB by mobilizing fat in the form of NEFA. In response to NEB, adipose tissue will be catabolized, therefore NEFA concentration will increase (McNamara, 1991), and some ruminants will start to mobilize NEFA pre-partum (Bertics et al., 1992). In contrast to ewes that were starved for 3 days, where the NEFA uptake and ketone output were relatively well matched; in the lactating animal the NEFA uptake and ketone production are no longer matched (Drackley and Andersen, 2006). This mis-match maybe related to the combination of increased growth hormone, decreased insulin, and decreased insulin to glucagon ratio which favors lipolysis. Elevated NEFA levels can be achieved by four mechanisms: suppression of de novo synthesis or uptake and then esterification of fatty acids; promotion of lipolysis; reduction of intracellular re-esterification of fatty acids released by lipolysis or a combination of all three (Bell, 1995).

The liver receives approximately one third of cardiac output and removes approximately 15 to 20% of the NEFA in circulation (Huntington et al., 1990; Drackley and Andersen, 2006). Once inside the liver, fatty acids have four pathways they can follow: complete oxidation in the TCA pathway to produce ATP; transported out of the liver in very low density lipoproteins (**VLDL**); used in beta-oxidation pathway to produce ketones or converted to ketones through peroxisomal oxidation; or stored in the liver as triglycerides (**TAG;** Drackley and Andersen, 2006). This review will focus on the effects of fat accumulation in the liver (hepatic lipidosis) and ketone production.

The ruminant liver has a very low capacity for exporting VLDL. There are 3 main proteins Apolipotrotein B_{100} (**$ApoB_{100}$**), Apolipoprotein E (**ApoE**), and microsomal triglyceride protiein (**MTP**) involved in the regulation of synthesis and secretion of VLDL. Although the

mechanisms by which these proteins are regulated have not been established, research has shown that $ApoB_{100}$ is down regulated and the other two proteins are upregulated (Bernabucci et al., 2004). The down regulation of $ApoB_{100}$ may be consistent with decreased synthesis and secretion of VLDL (Bernabucci et al., 2004) therefore TAG tend to accumulate in the liver (Herdt et al., 1983). Excessive accumulation of TAG in the liver impairs normal function (Rukkwamsuk et al., 1999; Jorritsma et al., 2001; Murondoti et al., 2004) such as decreased insulin clearance (Strang et al., 1998b) and decreased urea production, which may also indirectly decrease the rate of gluconeogenesis (Strang et al., 1998a). More recent research has evaluated the effect of elevated NEFA in vitro on the inhibition of hepatocyte gluconeogenesis (Li et al., 2012). Li et al., (2012) used quantitative PCR and spectrophotometry to show that when NEFA concentrations were higher than 0.5 and 1.5 mEq/L there was a marked decrease in mRNA levels of PC and PEPCK, respectively and when NEFA concentrations were higher than 1.5 and 0.5 mEq/L there was a marked decreased in enzyme activity for PC and PEPCK, respectively.

Hepatic lipidosis can severely compromise health and increase the risk of culling (Herdt, 1988). Although prior research has concentrated on the effects of fatty liver on the immune system, recent research has demonstrated that elevated levels of NEFA can also be detrimental to immune function (Contreras et al., 2010; Ster et al., 2012).

Ketogenesis

BHBA and NEB

As discussed previously, excessive mobilization of fatty acids during the transition period leads to production of ketones. Although the cause of hyperketonemia is not thoroughly understood, there are several theories proposed to help explain the progression of events.

Baird et al., (1968) presented the theory that the increased rate of gluconeogenesis during the transition period caused a decrease in OAA therefore an increase in ketogenesis; however, this theory was not supported. Ballard et al., (1968) found that there were no changes in PC or OAA levels in cows that developed spontaneous ketosis versus normal cows. Brown et al., (1992) hypothesized that ketogenesis was controlled by the principles of respiratory control such that substrate oxidation and ATP synthesis would proceed only as fast as needed to supply ATP for intracellular reactions, however, this was challenged by Berry et al., (1983) who demonstrated the reverse electron transport system in liver cells from rats, such that production of acetyl-CoA by beta-oxidation is obligatory linked to reverse electron transport. If this reverse electron system is correct, it would explain why ketones continue to be produced as NEFA concentration increase (Cadorniga-Valino et al., 1997), however, it seems unlikely that this mechanism could operate in ruminants. The role of carnitine palmitoyltransferase (**CPT I**) in regulating transport of LCFA into the mitochondria for beta-oxidation in ruminants has been reported by several authors (Brindle et al., 1985; Jesse et al., 1986; Drackley et al., 1991). However, Dann et al., (2004), reported that in periparturient dairy cows neither activity of CPT I or its inhibition by malonyl-CoA was related to an induced ketosis.

Drackley and Andersen (2006) reviewed the regulation of beta-oxidation and associated it with the disposal of acetyl-CoA in the mitochondria which can occur in three ways. Acetyl-CoA can be: 1. condensed with OAA to form citrate for complete oxidation of carbon dioxide in the TCA cycle; 2. used to form acetoacetyl-CoA in ketogenesis which results in release of acetoacetate (**AcAc**) that can be interconverted to BHBA with BHBA dehydrogenase; or 3. hydrolyzed by acetyl-CoA hydrolase which results in release of acetate from the mitochondria. Generally, it seems that ketone production seems to be a good avenue for utilization of the

acetyl-CoA derived from fatty acid beta-oxidation by helping decrease acetyl-CoA accumulation in the liver (Sato et al., 1999; Sugden et al., 2001). Conversion of acetyl-CoA to ketones allows the liver to oxidize five times as much NEFA with the same ATP production as if acetyl-CoA were completely oxidized in the TCA cycle (Drackley and Andersen, 2006).

Lastly, although the significance of peroxisomal beta-oxidation is controversial, this pathway may become more important when the mitochondrial beta-oxidative pathways are overloaded (Drackley and Andersen, 2006). Recent research has demonstrated that cows fed high energy diets during the transition period develop insulin resistance (Grummer, 1995; Holtenius et al., 2003; Dann et al., 2006) which can lead to unchecked lipolysis. These cows will have higher mobilization of adipose tissues, and in turn may overload the beta-oxidative pathways.

In the lactating animal with concurrent NEB, the increased oxidation of fatty acids in the liver results in a low cytosolic NADH : NAD ratio. Since conversion of AcAc to BHBA occurs in the cytosol, this ratio will impact the ratio of BHBA:AcAc released by the liver (Heitmann et al., 1987; Enjalbert et al., 2001). In contrast to fasting animals where the BHBA:AcAc ratio is low due to production rather than intercoversion of AcAc, lactating animals have a higher BHBA:AcAc ratio (Mills et al., 1986). These ratios are important when evaluating animals for hyperketonemia with different tests, such as milk ketone, urine, or blood. Enjalbert et al., 2001, used blood BHBA concentration at > 12 mg/dL as the gold standard to define SCK and compared: blood acetone to acetoacetate tests; milk acetone to acetoacetate, and milk BHBA tests. This study found that when these tests had high sensitivity (i.e., > 91%), but their specificities were low (57 to 84%). These sensitivities and specificities resulted in low predictive value positive (33.8 to 57.9%) and relatively high predictive value negative (97 to

98.4%). Additionally, when the correlation between tests (i.e., milk versus blood) was examined, the correlation coefficients were: milk acetone: blood acetone 0.96; milk AcAc: blood AcAc 0.74; and milk BHBA to blood BHBA 0.66.

The lack of correlation of ketone concentrations between blood and milk measurements will dictate the cut-points used to call a test positive. Recently, the Precision Xtra meter (Abott laboratories), a handheld device used to test blood BHBA concentrations was validated for use in ruminants. The sensitivity and specificity compared to serum BHBA concentrations determined photometrically are 96% and 98%, respectively, when using a cut-off value of $\geq$ 12 mg/dL (Iwersen et al., 2009; Konkol et al., 2009). There are other ketone tests on the market with varying degrees of sensitivity and specificity. For example: the Ketostix strip (Bayer Corporation, Elkhart, IN) evaluates acetoacetate in urine and when read after 5 seconds and interpreted as a "trace" had 90% sensitivity and 86% specificity and when interpreted as "small" has 78% sensitivity and 96% specificity both relative to serum at with BHBA $\geq$ 14 mg/dL (Carrier et al., 2004). The Ketotest (Sanwa Kagaku Co. Ltd., Nagoya, Japan) for milk when read at $\geq$ 5 mg/dL relative to serum at $\geq$ 14 mg/dL has 88% sensitivity and 90% specificity (Carrier et al., 2004).

Intervention strategies

Sustain glucose production

When an animal is experiencing excessive NEB, direct glucose administration will immediately provide glucose. Although the exogenous glucose may help initially, cows respond to glucose infusions by increasing glucose utilization in adipose and skeletal tissue (Al-Trad et al., 2009). Wagner et al., (2010) reviewed the effect of either 0.5 L or 1 L of 50% dextrose administration IV on NEFA, BHBA, glucose and insulin and reported that the activity of glucose

was short lived (<12 hours) and after 24 hours, NEFA and BHBA concentrations were back at subclinical ketosis levels. Insulin concentrations were increased in both treatment groups when compared to the control, but it was higher in the cows that received 1 L of 50% dextrose when compared to those that received just 0.5 L. In addition, dextrose treatment caused hypophosphatemia, which in early lactation cows, may exacerbate other mineral deficiencies. It is therefore more advantageous to provide glucose precursors in order to alleviate NEB like monensin or propylene glycol.

Ionophores, like monensin, increase glucose precursors because they alter rumen microbial populations such that microbes that produce propionate are over-represented in the rumen population (Bergen and Bates, 1984). These ionophores have been used in lactating cows to increase the glucogenic precursor and thus alleviate some of the negative downstream outcomes associated with excessive NEB (Duffield et al., 2008a; Duffield et al., 2008b; Duffield et al., 2008c; McGuffey et al., 2001). It is recommended to provide monensin starting a few weeks pre-partum until peak lactation (McGuffey et al., 2001).

Propylyne glycol (**PG**) has been known to provide glucogenic effects since the 1950s (Johnson, 1954), more recently, Kristensen et al., (2007) has demonstrated that an oral PG drench helps decrease glucose demand by peripheral tissues, despite the increase in insulin seen secondary to glucose increase. Currently, PG is used in cows in early lactation to help decrease concentrations of NEFA and BHBA (Overton and Waldron, 2004; McArt et al., 2011). More recently, McArt et al., (2011) showed that in cows that already had an increase in BHBA (BHBA $\geq$ 1.2 mg/dL) but were not yet clinically ketotic, benefited from PG administration and were less likely to develop clinical ketosis, more likely to resolve the SCK and produced more milk than the untreated controls.

Vitamin B12, was discussed previously, and has been used in conjunction with other ketosis treatments. Its effectiveness is likely related to the fact that MCM (the enzyme associated with gluconeogenesis from propionate) depends on Vitamin B12 (Aschenbach et al., 2010).

Minimize Hepatic Lipidosis

There are several feed additives that have been evaluated to help improve hepatic lipidosis by either improving the export of fat from the liver or decreasing TAG lipogenesis. Rumen-protected choline (**RPC**) may help hepatic export of fat in transition cows (Zom et al., 2011), and it may increase DMI and milk production, however, the optimal dose that will provide the best effect is still under investigation (Piepenbrink and Overton, 2003; Zahra et al., 2006; Zom et al., 2011). Zahra et al., (2006) reviewed the effects of monensin and rumen protected choline and reported that only cows with BCS > 4 had increased milk production. Niacin supplementation has also been evaluated because it can suppress lipolysis, however, dietary treatment is inconsistent and feeding too much can decrease DMI which can exacerbate NEB (Morey et al., 2011). Chromium potentiates the action of insulin and may also prevent lipolysis (Grummer, 2008); however, effects of chromium administration on metabolism were only modest (Smith et al., 2008). Although additional research on feed additives may help alleviate some of the excessive lipolysis and hepatic lipidosis, perhaps the most effective mechanism of managing lipolysis is to manage the BCS over the dry period. Overweight cows will have decreased DMI, elevated NEFA and BHBA concentrations post-partum, and are more likely to develop hepatic lipidosis (Holter et al., 1990; Van den Top et al., 1996).

General recommendations

Based on the previously reviewed literature, the current general management and nutritional recommendations to help improve the transition period is to have cows enter the dry period in good BCS, control energy demands during the dry period, minimize fat in the ration, minimize stress, maximize access to feed, and consider incorporating nutritional additives such as ionophores to help improve gluconeogenesis. However, even if these recommendations are followed, it is important to monitor cows in the transition period so that changes in management or nutrition can be made without having to wait for the negative downstream outcomes that associated with excessive NEB. In order to monitor transition cows, it is necessary to identify objective monitoring tools that are easy to use, inexpensive and accurate.

ASSOCIATION BETWEEN EXCESSIVE NEFA AND BHBA, AND NEGATIVE DOWNSTREAM OUTCOMES AT INDIVIDUAL ANIMAL LEVEL

Thus far, the evaluation of the transition period has focused on the process by which cows partition energy for lactogenesis despite having to enter a state of NEB. There are adaptive mechanisms in place to deal with NEB during the post-partum period, and the negative effects on health, reproduction and milk production are in fact associated with excessive NEB, and excessive elevation of NEFA or BHBA can indicate poor adaptation to NEB (Andersson, 1988; Herdt, 2000). The measurement and monitoring of these metabolites has been advocated by several authors (which will be reviewed below) and has proven useful when evaluating a transition cow program because of the association between elevated metabolite levels and negative downstream outcomes (Oetzel, 2004; Duffield et al., 2009).

Disease outcomes

As mentioned previously, both hepatic lipidosis and elevated NEFA and BHBA concentrations (Hoeben et al., 1997) have been associated with decreased immune function. Because cows are most likely to develop either an infection or metabolic disease early in lactation (LeBlanc et al., 2006), the association between excessive NEB and detrimental effects on the health of dairy cows during the transition period has been reviewed by several authors (Andersson, 1988; Kehrli et al., 1989; Hammon et al., 2006; Scalia et al., 2006).

Although the association between elevated concentration of the metabolites, NEFA and BHBA and detrimental effects was established, the critical threshold above which these detrimental effects were most likely was still under investigation. Cameron et al., (1998) reported that along with a high BCS, high energy in the dry cow diet, and reduced bunk space, NEFA concentration > 3 mEq/L between 35 and 3 d pre-partum was associated with an increased risk of displaced abomasum (**DA**) in 1170 multiparous cows in herd in Michigan. LeBlanc et al., (2005), evaluated over 1,000 cows in mostly tie-stall herds in Canada and reported that cows with pre-partum NEFA concentrations ≥ 0.5 mEq/L were 3.6 times more likely to develop a DA post-partum than those with NEFA concentrations < 0.5 mEq/L, and when BHBA and NEFA were measured in the post-partum period, only BHBA and not NEFA, was significantly associated with the risk of developing a DA. Cows with BHBA concentration ≥ 12 mg/dL were 8 times more likely to develop a DA. The methods for evaluating risk ratios (**RR**) versus odds ratios (**OR**), will be discussed in a later section, it is important to note that even though the previous study used logistic regression to evaluate the association between elevated metabolites and the development of a DA, the results were reported as RR. In cases where the outcome is not rare, such as studies that investigate mastitis, the OR can overestimate the RR resulting in over-estimation of the true effect.

Another study on 24 Canadian farms demonstrated that cows in the first week post-partum with elevated concentrations of BHBA (≥ 10 mg/dL) had 13.6 greater odds of developing a DA when compared to cows with BHBA below this concentration. Those with post-partum NEFA concentrations ≥ 1.0 mEq/L, and post-partum BHBA ≥ 12 mg/dL were associated with 6.3 and 4.7 greater odds of developing clinical ketosis, respectively (Seifi et al., 2011).

More recently, Chapinal et al., (2011) evaluated the association between elevated NEFA and BHBA and negative downstream outcomes in a large multi-center study on 2365 cows, by sampling the same cohort of cows from 1 week pre-partum to 1 week post-partum and then recording disease incidence. This group confirmed that cows with pre-partum NEFA concentrations ≥ 0.3 mEq/L and ≥ 0.5 mEq/L were more likely to develop retained placenta and metritis, respectively. When evaluating the odds of developing a DA they found that cows with pre-partum NEFA ≥ 0.5mEq/L and post-partum NEFA ≥1.0 mEq/L had increased odds. Pre-partum BHBA was also evaluated and although the specificity was low, they found that if pre-partum BHBA was ≥ 8.0 mg/dL, these cows had higher risk of developing a DA. This group evaluated both pre- and post-partum energy status, and found that the combined information of NEFA concentrations both pre- and post-partum were better predictors of disease risk than BHBA. The fact that information on NEFA pre- and post-partum was a better predictor than BHBA alone makes sense. Cows that are mobilizing fat reserves during the dry period are likely to have a more detrimental transition.

Although Chapinal et al., (2011) did not report on the ratio of NEFA to BHBA, an older study by Veenhuizen et al., (1991) reviewed NEFA and BHBA simultaneously. Veenhuizen et al., (1991) found that NEFA concentrations were increased 3, 2.6, 1.9 times at 3 weeks, 2 weeks, and at ketosis diagnosis and BHBA concentrations were increased 3.5, 5.8, and 8.4 times at the

same time points in cows with experimentally induced ketosis, thus the change in BHBA concentration was much more pronounced than NEFA at similar time points. Although studies with repeated measures may help us understand the physiology, the practical application to farms of this information may be limited. On modern farms, multiple samples on individual cows during the transition period may not be feasible, therefore research on the ratio of NEFA to BHBA at one sampling point and its relationship to negative downstream outcomes may be more useful.

Reproduction and milk production

When evaluating reproduction and milk production, previous reports suggests that there is an association between milk yield and decreased fertility; however, the results were controversial. Some studies reported that higher producing cows had lower fertility (Butler, 2003), whereas others did not (Hansen et al., 1983; Butler and Smith, 1989). This unexplained contradiction between milk yield and fertility coupled with the concept of homeorhesis focused the investigation toward energy balance deficits during the transition period and their effect on fertility. A subsequent study revealed that there was an association between excessive NEB and decreased reproductive performance; however, that study only examined BHBA concentrations, did not evaluate NEFA, and did not define a concentration threshold above which detrimental downstream outcomes were most likely (Walsh et al., 2007). Diminished reproductive effects may also be related to uterine disease (Reist et al., 2003; Hammon et al., 2006), and delayed luteal activity (Wathes et al., 2007), both of which have been shown to be associated with elevated BHBA concentrations. Excessive NEB has also been linked to decreased milk yield (Duffield et al., 2009). However, defining the level at which elevated NEFA or BHBA are

associated with milk production has been difficult because some elevation in these metabolites is characteristic of the transition period and because many other factors affect milk yield.

Although milk production is a sink for energy, it is important to note that with the exception of mastitis, milk production is not necessarily associated with increased risk of disease (Erb and Grohn, 1988; Cameron et al., 1998; Grohn, 2000; Ingvartsen et al., 2003). It is the individual animal response to these biological changes that dictate whether they will experience excessive NEB and thus be at higher risk of detrimental outcomes. Therefore it is important to explore the association between markers of NEB and milk production. Duffield et al., (2009) described an objective BHBA concentration threshold for the prediction of milk yield early in lactation, but the effects of pre- or postpartum NEFA concentrations were not evaluated. This group reported that cows with serum BHBA concentration $\geq$ 12 mg/dL measured during the first and second week post-partum made less milk.

STATISTICAL METHODS

Previous research which has evaluated the effects of elevated NEFA and BHBA during the transition has used statistical methods which estimated the OR. The OR is frequently used because it is the estimable measure of association in some study designs (i.e., case-control studies) or because estimating a more appropriate measure of association such as the RR was not readily feasible with commercially available statistical packages. Although the OR and not the RR was estimated, instead of reporting the odds, the results were sometimes misinterpreted as RR and incorrect phrases such as “more likely” or “risk” were used to describe the association between the risk factor(s) and the outcome of interest. Two problems are associated with estimating the OR in study designs where the RR can be estimated: (1) the OR can overestimate the true effect as the outcome becomes more common, and (2) interpretation of the OR is not

intuitive (Holcomb et al., 2001) because, unlike the RR, the OR does not directly measure effects on probability. In response to these concerns, recently statistical methods for estimating the RR have been proposed.

ASSOCIATION BETWEEN NEFA AND BHBA AND NEGATIVE DOWNSTREAM OUTCOMES AT THE HERD-LEVEL

Objective cow-level thresholds have been determined for increased BHBA and NEFA concentrations that are associated with disease, and reproduction, and production outcomes (LeBlanc et al., 2005; Duffield et al., 2009; Ospina et al., 2010; Ospina et al., 2010; Chapinal et al., 2011). This information allows the identification of individual cows at risk for these downstream outcomes based on their NEB status during the transition period. However, despite all of the NEB information available at the cow-level and its association with downstream outcomes, individual cow strategies for prevention of subclinical disease are still a challenge (Duffield, 2000). This is in part because changes associated with NEB adaptation can start as early as 6 wk pre-partum (Drackley et al., 2001). Efforts to improve NEB status should be implemented at the herd-level, where decisions about nutritional management and other aspects of the environment and herd management that in turn affect pre- and post-partum groups of cattle can be addressed appropriately. Unfortunately, information regarding the appropriate herd alarm levels (i.e., the proportion of sampled animals with increased concentrations of NEFA and BHBA) have not been well defined (Oetzel, 2004).

When evaluating excessive NEB at the herd-level, pooled samples may seem desirable due to lower laboratory cost. Although pooled samples are cost effective and useful in determining herd-level status for some infectious diseases, such as *Mycobacterium avium* subsp. *paratuberculosis* and Bovine Viral Diarrhea (Munoz-Zanzi et al., 2000; van Schaik et al., 2003), the ability to correctly determine herd-level NEB status through pooling has been questioned.

Pooled samples are good estimates of the arithmetic means of individual samples (Tornquist and Van Saun, 1999) but may be misleading when evaluating herd NEB status because individual animals may have very high concentrations of either metabolite and this will directly affect the pooled sample concentration. In addition, because animals have a normal baseline concentration for NEFA and BHBA and disease is associated when the metabolites are above a certain biological threshold (Oetzel, 2004) the arithmetic mean of the sample may not indicate that the herd has excessive NEB. Further evaluation of testing strategies at the herd-level is warranted.

CONCLUSIONS AND RESEARCH OBJECTIVES

The transition period is a very important time period for the dairy cow because, as discussed previously, the effects of excessive NEB can increase the risk of disease, can result in poor reproductive performance, and affect milk production. Being able to monitor cows at both the individual and herd-level will help farms manage this time period more effectively. Objective thresholds above which cows are more likely to have negative downstream outcomes are necessary in order to evaluate different programs, and perhaps more importantly, herd-alarm levels are necessary. Herd-alarm levels would allow a herd to monitor cows at the group level since this is the level where most management strategies take effect. Additionally, when a group of animals are evaluated it is important to address the idea of pooling samples. Pooling may help reduce the cost of sampling, but it may reduce sensitivity or specificity, thus this should be explored.

The objectives of this research were to:

1. Establish cow-level critical thresholds for NEFA and BHBA concentrations to predict key periparturient disease conditions and investigate the magnitude of the association of

these metabolites with disease conditions within 30 DIM in free-stall, TMR-fed herds in the northeastern United States.

2. Evaluate the association between elevated pre- and postpartum NEFA and BHBA concentrations on reproductive performance and milk production and to establish the metabolite concentrations above which the effects were most likely to occur.
3. Identify the herd alarm level for excessive NEB (i.e., the proportion of sampled animals with increased NEFA and BHBA) that was associated with herd-level changes in disease, reproduction and milk production.
4. Explain why the RR is the preferred measure of association when the outcome of interest is dichotomous in both cohort studies and randomized trials and to outline an applied method for estimating the RR using SAS version 9.2 (SAS Institute Inc., Cary, NC).
5. Compare the NEB status of a herd based on individual samples versus pooled samples using BHBA concentrations and estimate the herd level sensitivity and herd level specificity based on simulated individual animal sampling (varying sample size, underlying herd-level prevalence of elevated BHBA, and test sensitivity and specificity), and 3) estimate the herd predictive value positive and herd predictive value negative under the same simulation and discuss the frequency of sampling.

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CHAPTER TWO

EVALUATION OF NEFA AND B-HYDROXYBUTYRATE (BHBA) IN TRANSITION DAIRY CATTLE IN THE NORTHEAST USA. CRITICAL THRESHOLDS FOR PREDICTION OF CLINICAL DISEASES

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ABSTRACT

The objectives of this study were to 1) establish cow- level critical thresholds for serum concentrations of non-esterified fatty acids (**NEFA**) and beta-hydroxybutyrate (**BHBA**) to predict periparturient diseases [displaced abomasa (**DA**), clinical ketosis (**CK**), metritis and retained placenta, or any of these three], and 2) investigate the magnitude of the metabolites' association with these diseases within 30 d in milk. In a prospective cohort study of 100 freestall, total mixed ration-fed herds in the northeastern United States, blood samples were collected from approximately 15 pre-partum and 15 different post-partum transition animals in each herd, for a total of 2,758 samples. Serum NEFA concentrations were measured in the pre-partum group, and both NEFA and BHBA were measured in the postpartum group. The critical thresholds for NEFA or BHBA were evaluated with receiver operator characteristic analysis for all diseases in both cohorts. The risk ratios (RR) of a disease outcome given NEFA or BHBA concentrations and other covariates were modeled with multivariable regression techniques, accounting for clustering of cows within herds. The NEFA critical threshold that predicted any of the 3 diseases in the pre-partum cohort was 0.29 mEq/L and in the postpartum cohort was 0.57 mEq/L. The critical threshold for serum BHBA in the post-partum cohort was 10 mg/dL, which predicted any of the 3 diseases. All RR with NEFA as a predictor of disease were > 1.8; however, RR were greatest in animals sampled postpartum (e.g., RR for DA = 9.7; 95% CI = 4.2 to 22.4. All RR with BHBA as the predictor of disease were > 2.3 (e.g., RR for DA = 6.9; 95% CI = 3.7 to 12.9). Although pre-partum NEFA and postpartum BHBA were both significantly associated with development of clinical disease post-partum serum NEFA concentration was most associated with the risk of developing DA, CK, metritis or retained placenta during the first 30 d in milk.

Keywords: dairy cow, nonesterified fatty acids, β-hydroxybutyrate, disease

INTRODUCTION

Most transition dairy cows enter a state of negative energy balance (NEB) for 3 primary reasons: increased energy demands at parturition, decreased DMI shortly before parturition, and lagging DMI compared with energy demands due to milk production (Gerloff, 2000; Hayirli et al., 2002). The energy need of a transition cow increases from approximately 1 kg/d of glucose during late gestation to 2.5 kg/d during the first 3 wk post-calving (Reynolds et al., 2003). Because of increased energy demand and decreased DMI, the transition cow must meet this challenge by mobilizing adipose tissue. Some degree of NEB, which can be identified by an increase in circulating concentrations of NEFA and BHBA, is expected in the transition period as the cow adjusts to new energy demands and energy intake catches up with production. Stored energy from fat is mobilized as NEFA, some of which is taken up by the liver. In the liver some NEFA are oxidized or reesterified into triglycerides that are either exported as very low density lipoproteins or stored in the liver. During the periparturient period, high rates of NEFA enter the liver and sometimes exceed the liver's capacity to secrete triglycerides as very low density lipoproteins, resulting in an accumulation of triglycerides (Drackley et al., 2001). Increased amounts of NEFA removed by the liver along with carnitine palmitoyltransferase-1 activity regulate ketogenesis and thus, BHBA production (Hegardt, 1999).

Excessive NEB (ENEB) had detrimental effects on the health and production of dairy cows because of the suspected relationship between energy deficiency and immunosuppression (Kehrli et al., 1989; Hammon et al., 2006; Scalia et al., 2006). Although a study in Ontario reviewed the association between ENEB and metabolic and reproductive disease in transition cows, that study focused on smaller, usually component-fed herds (Duffield et al., 1999). The ability to predict at the cow level which animals are more likely to develop disease based on NEFA and BHBA concentrations might help producers prevent diseases proactively by focusing

on management and nutritional strategies to prevent subclinical and clinical disease. The objectives were to 1) establish cow-level critical thresholds for NEFA and BHBA concentrations to predict key periparturient disease conditions, and 2) investigate the magnitude of the association of these metabolites with disease conditions within 30 DIM in free-stall, TMR-fed herds in the northeastern United States. The periparturient conditions investigated were 1) displaced abomasum (DA), 2) clinical ketosis (CK), 3) either retained placenta (RP) or metritis (MET) or both, or 4) any of these 3 disease conditions.

MATERIALS AND METHODS

Study Population and Study Design

A prospective cohort study was conducted from a convenience sample of 104 dairy herds in New York, Pennsylvania, and Vermont between January 2006 and July 2007. Inclusion criteria for herds were 1) >250 milking cows, 2) free-stall housing, 3) fed a TMR, and 4) participated in DHIA or used Dairy Comp 305 (version 2009; Valley Ag Software, Tulare, CA) or both.

A convenience sample of apparently healthy heifers and cows in the transition period were selected. At the time of sample collection, healthy heifers or cows were defined as not being in the sick pen, not currently receiving any medical treatment, and not displaying sick cow behavior based on the subjective interpretation of the research staff. At sample collection, 2 cohorts of animals were identified: those 14 to 2 d prepartum and those 3 to 14 d postpartum. In each herd, cross-sectional sampling of approximately 15 animals from each group was done to achieve approximately 90% confidence of within-herd prevalence. To reflect common herd demographics, approximately one-third of the animals sampled were primiparous (both before and after first parturition).

Animals were followed through 30 DIM for incidence of the selected periparturient diseases. In the cohort sampled prepartum, the diseases of interest were DA, CK, and MET or RP, a combination of RP and MET, and any combination of the 3. In the cohort sampled postpartum, diseases of interest were DA, CK, MET, and any combination of these 3. Metritis and RP were evaluated as one disease in animals sampled prepartum because of possible misclassification of metritis. Retained placenta was not evaluated in the cohort sampled postpartum because cows sampled at 3 to 14 DIM were no longer at risk for RP.

Farm Survey and Case Definitions

Efforts were made to limit differences between farms on all levels of data collection. All farmers received a standardized consent form, survey, and case definitions for diseases of interest. All farmers consented to participate, and the study was approved by the Cornell University Institutional Animal Care and Use Committee. The survey included demographic information, and feeding times in relation to blood collection (Eicher et al., 1999) to be used as potential risk factors. Farm personnel were instructed to document any incident cases of diseases both on the farm survey and in Dairy Comp 305. Specifically, they were to document cases of DA, CK, and MET or RP.

For consistency of documentation, the diseases were defined and case definitions were provided to farm personnel: 1) DA = movement of the fourth compartment of the stomach to a location on the right (RDA) or left side (LDA) of the cow and detected by ausculting a “ping” sound with finger percussion. Often, a cow had an abrupt decrease in milk production and was off feed; 2) CK = cow that was off feed, had sudden weight loss, and decreased milk production, but had no other detectable signs of disease and was treated with dextrose, propylene glycol, steroids, or a combination (Duffield et al., 1999); 3) MET = sick cow (dull, decreased milk yield)

that had a fever greater than 39.5°C with a fetid (purulent or red to brown color or both) discharge from the vulva and was <21 DIM (Sheldon et al., 2006); and 4) RP = failure to expel fetal membranes within 24 h after calving.

Blood Collection and Analysis

Blood samples were collected from each cow and BCS were assigned (Ferguson et al., 1994) concurrently with blood sample collection. Guidelines for blood collection and sample handling were based on Stokol and Nydam (2005). Briefly, a plain evacuated red-top tube was used to collect 10 mL of blood from the coccygeal vein or artery. The blood was stored in a cooler (4°C), separated from cells within 24 h, and analyzed at the Cornell Animal Health Diagnostic Center (Ithaca, NY) within 48 h of collection. All samples were analyzed using an automated wet chemistry analyzer (Hitachi 917, Roche Diagnostics, Indianapolis, IN). The sera from the pre-partum cohort were analyzed for NEFA (NEFA-C, Wako Chemicals USA Inc., Richmond, VA) and hemolysis (Stokol and Nydam, 2006). The sera from animals sampled postpartum were analyzed for NEFA, BHBA (β-HB, Catachem Inc., Bridgeport, CT), and hemolysis.

Individual animal observations were excluded for the following reasons: the sample was severely hemolysed (Stokol and Nydam, 2006) or day of sample collection was out of sampling range for inclusion; for example, animals that were >14 d prepartum when sampled or >14 DIM at the time of sample collection.

Statistical Analysis

Multivariable Analysis. Concentrations of NEFA and BHBA were the main risk factors of interest in the evaluation of the development of any combination of the diseases. At this level of analysis, the metabolites NEFA and BHBA were treated as continuous variables. The other

covariates considered were parity, season, BCS, time of blood collection in relation to feeding, and all biologically plausible 2-way interactions. They were modeled with PROC GENMOD using a Poisson distribution, a log link function, p-scale option for over-dispersion, and an exchangeable correlation matrix (Spiegelman and Hertzmark, 2005). There was no adjustment for varying time spans (offset term) because the length of the time interval was the same for every individual in the sample (Allison, 1999). This statistical method allowed for clustering of cows within herds (i.e., including herd as a random effect) while adjusting for continuous or categorical covariates.

Three full models (1 for animals sampled prepartum and 2 for animals sampled postpartum) were evaluated to predict the development of any combination of the diseases. The models were 1) prepartum NEFA, covariates, and biologically plausible 2-way interactions between main effects and covariates, 2) postpartum NEFA, BHBA, covariates, and 2-way interactions, and 3) BHBA, covariates, and 2-way interactions in animals sampled postpartum. Beta-Hydroxybutyrate is easily measured on dairy farms using point-of-care analyzers; therefore, its effect was evaluated in a model without NEFA. Covariates and interactions that were not significant at $P > 0.10$ were removed by manual backward stepwise elimination.

Receiver Operator Characteristic Analysis for Critical Thresholds.

The continuous variables that remained in the final multivariable model were evaluated with receiver operator characteristic (ROC) analysis to determine a critical threshold for predicting disease. The ROC curves analyze sensitivity versus 100 - specificity. Sensitivity was the proportion of animals diagnosed with disease that were above a given metabolite threshold, and specificity was the proportion of animals that did not have the diseases that was below a given threshold (Greiner et al., 2000). The point on the ROC curve that had the highest combined

sensitivity and specificity was considered the critical threshold. Interpretation of this critical threshold was based on the area under the curve (AUC) such that if the AUC = 0.5 it was non-informative; if $0.5 < AUC \leq 0.7$, it was accurate; if $0.7 < AUC \leq 0.9$, it was very accurate; if $0.9 < AUC < 1$, it was highly accurate; and if AUC = 1, then it was considered perfect (Swets, 1988). The significant predictor variables from the multivariable analysis were analyzed using ROC curves to determine the critical thresholds for both individual diseases and any combination of those diseases.

Likelihood ratios (LR) were evaluated. The LR positive (LR+) was the probability that a test result at or above the threshold would be more likely to come from an animal later diagnosed with disease.

Measures of Association.

Risk ratios (RR) were modeled with PROC GENMOD, with a Poisson distribution, a log link function, p-scale option for over-dispersion adjustment, and an exchangeable correlation matrix (Spiegelman and Hertzmark, 2005). At this level of analysis the significant covariates, NEFA and BHBA, were treated as categorical variables based on the thresholds from ROC analysis.

Statistical analyses of data were conducted using SAS version 9.1 (SAS Institute, 2004), and ROC curves were obtained using MedCalc version 9.5.2.0 (Schoonjans, Mariakerke, Belgium). All data were stratified based on prepartum or postpartum status at time of sample collection.

RESULTS

Descriptive Data

Of the 104 herds, 4 were excluded from the study because of missing data. There were 2,758 cows from the remaining 100 herds included and of these cows, 1,440 were sampled prepartum (35% heifers and 65% cows) and 1,318 were sampled postpartum (37% heifers and 63% cows). The number of milking cows per herd ranged from 265 to 2,770, with a median of 767 and mean of 840.

In animals sampled pre-partum, the lactational incidence of each disease condition was as follows: DA, 3.3%; CK, 7.0%; MET or RP, 12.1%; and any combination of these 3 conditions, 19.6%. In animals sampled postpartum, the lactational incidences were as follows: DA, 3.1%; CK, 4.6%; MET, 2.9%; and any combination, 9.0%. The median DIM at diagnosis for diseases in animals sampled pre-partum were DA 10, CK 7, MET 5, and RP 2. The median DIM at diagnosis for diseases in animals sampled post-partum were DA 15, CK 11, and MET 9. The median BCS for heifers sampled pre-partum was 4 (range: 2 to 4.75) and for cows 3.5 (2 to 4.5). The median BCS for heifers sampled post-partum was 3.5 (2 to 4.5) and for cows 3.25 (1.75 to 4.5).

Multivariable Analysis

In the multivariable model for animals sampled pre-partum, NEFA ($P = 0.03$) was the only predictor retained in the model, and there were no interactions with $P < 0.16$. In the postpartum multivariable model with NEFA ($P = 0.0005$) and BHBA ($P = 0.29$), NEFA was the only predictor retained. When, in a third model, BHBA ($P = 0.005$) was modeled as the main effect without NEFA, it was the only predictor retained and no interactions had a $P < 0.16$.

Critical Thresholds

Nonesterified fatty acids and BHBA were the only significant predictors identified in the multivariable models. They were then analyzed with ROC curves to determine the cow-level critical thresholds (combined highest sensitivity and specificity) to predict individual diseases as well as any combination of the diseases. Tabular results of ROC curve determination of critical NEFA (mEq/L) thresholds for the prediction of individual diseases as well as the combination of diseases are in Table 2.1.

In summary, for the prediction of the development of any disease, the pre-partum NEFA critical threshold was 0.29 mEq/L and the postpartum NEFA threshold was 0.57 mEq/L. Figure 2.1 is a graphical example of a ROC curve with NEFA as a predictor of DA in animals sampled postpartum. As a test for the prediction of DA, additional information on various levels of NEFA are in Table 2.2, showing sensitivity, specificity, and LR for various levels of pre-partum and postpartum NEFA used for comparison with other studies and to provide additional information to readers.

Results for BHBA concentrations (mg/dL) are reported similarly. Table 2.3 identifies the critical BHBA thresholds in animals sampled post-partum when predicting individual diseases as well as any of the diseases. Briefly, the critical threshold for predicting any disease was 10 mg/dL. Table 2.4 provides additional information on various levels of BHBA as a test for prediction of DA. Likelihood ratios were calculated based on critical thresholds determined by univariable ROC analysis and reported in Tables 2.1 to 2.4. In general, the LR+ reports the probability that a test result at or above a given threshold will have a greater chance of coming from an animal later diagnosed with disease. For example, the post-partum NEFA optimal threshold for predicting DA was 0.72 mEq/L; this resulted in 3.0 LR+. The interpretation is that

a NEFA test value at or above this threshold (0.72 mEq/L) was 3 times as likely to come from a cow that was later diagnosed with DA.

Measures of Association

Risk ratios were calculated using multivariable modeling after critical thresholds were determined in ROC analysis (Tables 2.5 and 2.6). All RR were significant and >1.8. In the cohort sampled pre-partum, all risk ratios were >1.8 (range: 1.8 to 2.2; Table 5), meaning, for example, that the risk of getting any of the diseases later was at least 1.8 times greater in cows with a NEFA concentration higher than the threshold value (0.29 mEq/L). In the cohort sampled postpartum, all NEFA risk ratios were >4.4 (range: 4.4 to 17). An example of one of the larger risk ratios is the one associated with developing a DA, where cows with postpartum NEFA ≥0.72 mEq/L were almost 10 times more likely to develop a DA within 30 DIM. Risk ratios based on BHBA concentrations were significant and >2.3 (2.3 to 6.9, Table 2.6) meaning that there was a higher risk of developing disease if animals had a BHBA level higher than the threshold.

Table 2.1 Receiver operator characteristic (ROC) curve determination of critical Non-esterified Fatty Acids (NEFA) thresholds as predictors of disease in transition dairy cows.

Disease	Critical threshold[1]	Sen[2]	95% CI[3] for Sen	Spec[4]	95% CI for Spec	LR +[5]	AUC[6]	*P*
Animals sampled pre-partum (n = 1440)								
DA	0.27	57	42 to 72	62	60 to 65	1.5	0.6	0.01
CK	0.26	53	43 to 64	61	58 to 64	1.4	0.6	0.001
MET or RP or both	0.37	37	30 to 45	80	78 to 83	1.9	0.6	0.0001
Any 3	0.29	48	42 to 54	69	67 to 72	1.6	0.6	< 0.0001
Animals sampled post-partum (n = 1318)								
DA	0.72	80	65 to 91	73	70 to 75	3.0	0.8	< 0.0001
CK	0.57	74	61 to 84	59	57 to 62	1.8	0.7	< 0.0001
MET	0.36	97	86 to 100	30	28 to 33	1.4	0.6	0.009
Any 3	0.57	75	66 to 82	61	58 to 64	1.9	0.7	< 0.0001

[1] Highest combined specificity and sensitivity, mEq/L
[2] Sen = epidemiologic sensitivity
[3] CI = Confidence Interval
[4] Spec = epidemiologic specificity
[5] Likelihood ratio positive
[6] AUC = Area under the curve

Table 2.2. Additional information on cut-points from receiver operator characteristic (ROC) curves for Non-esterified Fatty Acids (NEFA) concentrations as predictors of DA.

Thresholds (mEq/L)	Sensitivity	95% CI[1] for Sensitivity	Specificity	95% CI for Specificity	LR +[2]
Animals sampled pre-partum (n = 1440)					
0.2	64	49 to 73	48	45 to 51	1.2
0.27[3]	57	42 to 72	62	60 to 65	1.5
0.4	30	17 to 45	82	80 to 84	1.7
0.5	23	12 to 38	89	87 to 91	2.2
Animals sampled post-partum (n = 1318)					
0.4	95	83 to 99	39	37 to 42	1.6
0.72[3]	80	65 to 91	73	70 to 75	3.0
1.02	59	42 to 74	87	85 to 89	4.6

[1] CI = Confidence interval
[2] Likelihood ratio positive
[3] Highest combined sensitivity and specificity in this study

Table 2.3. Receiver operator characteristic (ROC) curve determination of critical β-hydroxybutyrate (BHB) thresholds as predictors of disease in transition dairy cows.

Animals sampled post-partum (n = 1318)								
Disease	Critical threshold [1]	Sen[2]	95 % CI[3] for Sen	Spec[4]	95 % CI for Spec	LR +[5]	AUC[6]	*P*
DA	10	71	55 to 84	80	77 to 82	3.5	0.8	< 0.0001
CK	10	57	44 to 70	80	78 to 82	2.8	0.7	< 0.0001
MET	7	63	46 to 78	59	56 to 61	1.5	0.6	0.03
Any 3	10	57	47 to 66	82	79 to 84	3.1	0.7	< 0.0001

[1] Highest combined specificity and sensitivity, mg/Dl
[2] Sen = Epidemiologic sensitivity
[3] CI = Confidence Interval
[4] Spec = Epidemiologic specificity
[5] Likelihood ratio positive
[6] AUC = Area under the curve

Table 2.4. Additional information on cut-points from receiver operator characteristic (ROC) curves for β-hydroxybutyrate (BHB) concentrations as predictors of DA in animals sampled post-partum.

Animals sampled post-partum (n = 1318)					
Threshold (mg/dL)	Sensitivity	95 % CI[1] for Sensitivity	Specificity	95 % CI for Specificity	LR +[2]
8	76	60 to 88	69	67 to 72	2.5
10[3]	71	55 to 84	80	77 to 82	3.5
12	63	47 to 78	86	84 to 88	4.6
14	51	35 to 67	90	88 to 91	4.9

[1]Confidence Interval
[2] Likelihood ratio positive
[3] Highest combined sensitivity and specificity

Table 2.5. Risk ratios of disease based on Non-esterified Fatty Acids (NEFA) critical thresholds derived from ROC curve analysis.

Disease	Critical threshold (mEq/L)	Estimate	SE[1]	*P*[2]	RR[3]	95% CI[4]
Animals sampled pre-partum (n = 1440)						
DA	0.27	0.68	0.32	0.03	2.0	1.1 to 3.7
CK	0.26	0.56	0.18	0.001	1.8	1.2 to 2.5
MET or RP or both	0.37	0.78	0.17	< 0.0001	2.2	1.6 to 3.0
any of the 3	0.29	0.56	0.12	< 0.0001	1.8	1.4 to 2.2
Animals sampled post-partum (n = 1318)						
DA	0.72	2.3	0.43	< 0.0001	9.7	4.2 to 22
CK	0.57	1.6	0.41	< 0.0001	5.0	2.3 to 11
MET	0.36	2.8	1.1	0.008	17	2 to 134
any of the 3	0.57	1.5	0.26	< 0.0001	4.4	2.6 to 7.3

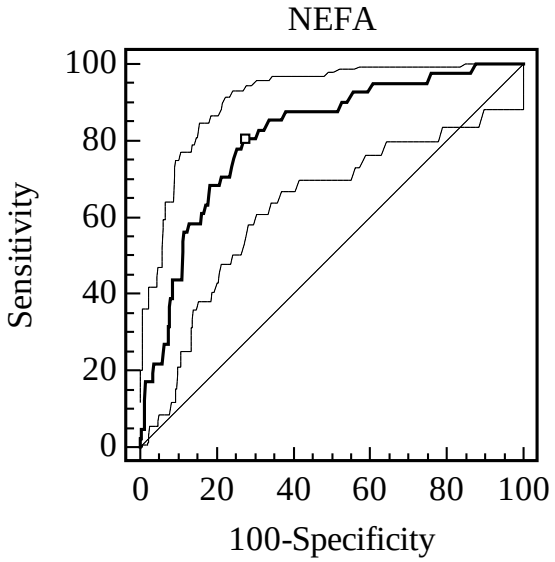
[1] SE = Standard error for estimate
[2] P-Value reported for estimate
[3] RR = Risk ratio
[4] CI = Confidence interval for RR

Table 2.6. Risk ratios of disease based on post-partum β-hydroxybutyrate (BHB) critical thresholds derived from ROC curve analysis.

Disease	Critical threshold (mg/dL)	Estimate	SE[1]	*P*[2]	RR[3]	95% CI[4]
Animals sampled post-partum (n =1318)						
DA	10	1.9	0.32	< 0.0001	6.9	3.7 to 12.9
CK	10	1.6	0.21	< 0.0001	4.9	3.2 to 7.3
MET	7	0.85	0.41	0.04	2.3	1.1 to 5.2
any of the 3	10	1.5	0.18	< 0.0001	4.4	3.1 to 6.3

[1] SE = Standard error for estimate
[2] Values reported for estimate
[3] RR = Risk ratio
[4] CI = Confidence Interval for RR

Figure 2.1. ROC curve determination of critical threshold (upper most left hand corner □) for Non-esterified Fatty Acids (NEFA) concentrations (≥ 0.72 mEq/L) predicting DA in animals sampled post-partum. The diagonal line represents the sensitivity and specificity level at which the test is non-informative.



DISCUSSION

The analytical approach used to examine the association between both pre- and postpartum NEFA and postpartum BHBA and the development of several diseases in transition cows allowed reporting the RR directly rather than approximating it with odds ratios. The ROC curves were used to determine the critical thresholds of the metabolites as predictors of diseases in TMR-fed, free-stall herds milking an average of 840 cows. The results generally support other reports of transition cow disease: elevated NEFA and BHBA concentrations were associated with the development of the diseases of interest (DA, CK, MET or RP). This association was reported by Dohoo and Martin (1984) who examined the association between subclinical ketosis and production and disease. Cameron et al., (1998) and LeBlanc et al., (2005) explored this association with DA as the outcome and determined that elevated metabolite levels were

predictive of disease. Duffield et al., (2002) reported that improved energy metabolism reduced the incidence of DA and RP, confirming the association between the 2 factors. Kaneene et al., (1997) and Oetzel (2004) reported similar relationships.

Concentrations of NEFA and BHBA were the main risk factors for predictors of disease after controlling for the random effect of herd, parity, season, BCS, sample collection time before feeding, and all biologically plausible 2-way interactions. There were no significant interactions between the main effects and other covariates. Neither BCS nor its interaction with metabolites was significant. This is not surprising because it was the loss of body condition that was most closely related to problems with ENEB (Busato et al., 2002; Kim and Suh, 2003). Beta-Hydroxybutyrate concentrations fluctuate throughout the day and are generally highest 4 to 5 h after feeding, whereas NEFA concentrations are generally highest before feeding (Oetzel, 2004). Previous studies show that NEFA is less sensitive to time of sample collection, whereas BHBA is more sensitive (Eicher et al., 1999). In this study, the interaction between the metabolites NEFA and BHBA and the timing of blood collection relative to feeding were not significant. Days in milk or number of days before parturition were not included as covariates because the objective was to identify the critical threshold for the specified time frame, not describe the difference in metabolite levels based on DIM or days until parturition.

In general, LR+ increased as metabolite levels increased. Biologically this is logical because circulating NEFA and BHBA serum concentrations can be used as markers of energy status. Therefore, levels above a given threshold should have a positive relationship with the probability of developing disease (i.e., higher metabolite levels are more predictive of disease). The LR related to DA and prepartum NEFA (LR = 1.5), postpartum NEFA (LR = 3), and BHBA (LR = 3.5) supported results from other studies. For example, LeBlanc et al., (2005)

reported that a prepartum NEFA test result ≥0.5 mEq/L was twice (LR = 1.9) as likely to come from a cow later diagnosed with LDA as from one without an LDA; similarly they reported that a postpartum NEFA test result ≥1.0 mEq/L was 3.5 times as likely to come from a cow later diagnosed with an LDA. In Duffield et al., (2009), test results of BHBA concentration ≥12 mg/dL measured 1 wk postpartum were 1.87 times as likely to come from a cow later diagnosed with LDA. Yet, in comparison to LR where there was a high prior probability of having the condition in question, these LR were low.

Risk ratios were reported as measures of association between the metabolites (NEFA or BHBA) and disease outcomes. An RR >1 indicated that animals with metabolite levels above the critical threshold (exposed group) were at higher risk for development of the disease than the animals below the critical threshold (unexposed group). When NEFA was evaluated as predictor of DA in animals sampled postpartum, the risk ratio was 9.7; that is, animals sampled between 3 to 14 DIM with a NEFA level ≥0.72 mEq/L were approximately 10 times more likely to develop a DA than animals below this threshold. In general, postpartum NEFA concentrations resulted in the largest RR compared with those reported for prepartum NEFA and BHBA in this and other studies (Kaneene et al., 1997; Cameron et al., 1998; LeBlanc et al., 2005). When BHBA was evaluated as the main predictor of disease, all RR were significant.

The critical thresholds at which the metabolites were predictive of disease were lower than in previous reports. Some previous studies sampled over different time frames and focused on different populations, often smaller, component-fed herds. LeBlanc et al., (2005) sampled cows 10 to 4 d prepartum, in small, often component-fed herds and reported that the critical threshold for predicting an LDA with NEFA was at ≥0.5 mEq/L, with an odds ratio of 3.6. They found that animals sampled up to 1 wk postpartum with NEFA ≥1 mEq/L yielded an odds ratio of

4.8; and a BHBA concentration of ≥12 mg/dL resulted in an odds ratio of 8. Kaneene et al, (1997) did not report a critical value for NEFA or BHBA, but animals sampled postpartum (3 to 35 DIM) presented a probable association between metabolic events associated with energy insufficiency and the risk of MET and RP. In high-producing Michigan dairies, Cameron et al., (1998) sampled animals 3 to 35 d prepartum and found that animals with NEFA >0.3 mEq/L were twice as likely to develop an LDA. Sampling from the first week postpartum, Geishauser et al., (2000) reported that the odds of developing a LDA were 4 times higher in animals with BHBA levels ≥14 mg/dL. They reported that if BHBA was at this level in the second week postpartum, the odds were 8:1 that animals would develop an LDA.

There were some limitations to this study including possible disease misclassification and loss to follow-up. Although several steps were taken to prevent disease misclassification (e.g., case definitions and careful monitoring), MET may not have been properly diagnosed in all groups. Metritis can be difficult to diagnose, especially if it coincides with RP. Cases of CK may have been misclassified because ketone levels were not directly measured. Loss to follow-up is a limitation inherent to prospective cohort studies. A small degree of loss to follow-up was experienced because cows from 4 farms were excluded due to missing cow disease information. At the cow level in the postpartum cohort, cows that were sick at time of sample collection were not eligible to be part of the study. This may have influenced the median DIM at disease diagnosis. In addition, as many cows as intended were not sampled in all herds for several reasons: smaller herds did not have enough eligible cows at time of sampling and some samples were discarded because of hemolysis.

Postpartum NEFA had a higher association with the development of disease than did prepartum NEFA or postpartum BHBA as reflected by larger RR. This association suggested

that the energy status as measured by NEFA from animals sampled postpartum (3 to 14 DIM) may have a more direct association with the development of disease than their energy status measured by BHBA or prepartum NEFA. When compared with prepartum NEFA, time of sample collection may play a role: samples collected postpartum were temporally much closer to the disease event and were perhaps better able to predict this event. Postpartum NEFA concentration as a predictor of disease has not been investigated as thoroughly as BHBA concentration or prepartum NEFA concentration. The AUC values from ROC analysis coupled with larger risk ratios suggested that postpartum NEFA could be used similarly to BHBA and prepartum NEFA

CONCLUSION

The effects of elevated concentrations of NEFA and BHBA in the transition period predicted clinical disease (e.g., DA, CK, MET, or RP) in cattle from TMR-fed northeastern US free-stall dairies with an average of 840 cows. The following cow-level critical thresholds should be considered general guidelines for monitoring cattle: NEFA concentrations ≥0.3 mEq/L for cattle 14 to 2 d prepartum; and NEFA concentrations ≥0.6 mEq/L and BHBA ≥10 mg/dL for cattle 3 to 14 d postpartum. Both pre- and postpartum NEFA concentrations and BHBA concentrations above these critical thresholds were associated with increased risk for subsequent disease.

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CHAPTER THREE

ASSOCIATIONS OF ELEVATED NONESTERIFIED FATTY ACIDS AND BETA-HYDROXYBUTYRATE CONCENTRATIONS WITH EARLY LACTATION REPRODUCTIVE PERFORMANCE AND MILK PRODUCTION IN TRANSITION DAIRY CATTLE IN THE NORTHEASTERN UNITED STATES

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ABSTRACT

The objectives were to evaluate the effects of elevated pre- and post-partum non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA) concentrations during the transition period on reproductive performance and milk production in dairy cattle. In a prospective cohort study of 91 freestall, total mixed ration-fed herds in the northeastern United States, blood samples were collected from approximately 15 pre-partum and 15 different post-partum transition animals in each herd. All samples were stratified based on pre- or post-partum status at the time of sample collection, and 2,259 and 2,290 animals were used to evaluate reproductive and milk production performance, respectively. Reproductive performance was assessed by time to conception within 70 d post-voluntary waiting period (VWP) and milk production was assessed using mature-equivalent 305-d (ME305) milk yield estimated at 120 d in milk. While controlling for body condition score (BCS), calving season, median ME305 milk production, and parity, NEFA and BHBA concentrations were evaluated with time to event analysis to investigate reproductive performance. These same predictor variables were used to determine the effects of elevated NEFA and BHBA concentrations on ME305 milk yield with herd as a random effect. Heifers and cows were grouped in the final analyses if the results between groups were similar. In all animals sampled pre-partum, the risk of pregnancy within 70 d post-VWP was reduced by 19% when NEFA concentrations were ≥0.27 mEq/L. In all animals sampled post-partum, those with NEFA concentrations ≥0.72 mEq/L had a 16% decrease in risk of pregnancy and those with BHBA concentrations ≥10 mg/dL had a 13% decrease in risk. In cows and heifers, ME305 milk yield was decreased by 683 kg when pre-partum NEFA concentrations were ≥0.33 mEq/L. In heifers sampled post-partum, ME305 milk yield was increased by 488kg when NEFA concentrations were ≥0.57 mEq/L and increased by 403 kg when BHBA concentrations were ≥9mg/dL. In cows sampled post-partum, ME305 milk

yield was decreased by 647 kg when NEFA concentrations were ≥0.72 mEq/L and decreased by 393 kg when BHBA concentrations were ≥10 mg/dL. With the exception of milk production in heifers, this study indicates that increased concentrations of serum NEFA and BHBA had a detrimental effect on reproductive performance and milk production.

Key words: nonesterifiedfattyacids, beta-hydroxybutyrate, milk production, pregnancy

INTRODUCTION

The ability of dairy cattle to partition available energy for milk production at the expense of reproduction early in lactation has made the role of energy balance a key factor in the study of reproductive performance and milk production. The concept of prioritization of energy and other nutrients, also known as homeorhesis, was described in 1980 (Bauman and Currie, 1980) and has set the foundation for the study of negative energy balance (**NEB**) in transition dairy cattle. The circulating metabolites non esterified fatty acids (**NEFA**) and beta-hydroxybutyrate (**BHBA**) are commonly used indices of NEB or ketosis in transition animals. Although some elevation of these metabolites is normal as these animals balance energy intake and energy demands in early lactation, excessive elevation of NEFA or BHBA can indicate poor adaptation to NEB (Herdt,2000).

Previous reports suggest that there is an association between milk yield and decreased fertility; however, the results were controversial. Some studies reported that higher producing cows had lower fertility (Butler,2003), whereas others did not (Hansen et al., 1983b; Butler and Smith, 1989). This unexplained contradiction between milk yield and fertility coupled with the concept of homeorhesis focused the investigation toward energy balance deficits during the transition period and their effect on fertility. Subsequent studies revealed that there was an association between excessive NEB and decreased reproductive performance (Walsh et al., 2007); however, that study only examined BHBA concentrations, did not evaluate NEFA, and

did not define a concentration threshold above which detrimental downstream outcomes were most likely. Diminished reproductive effects may also be related to uterine disease (Reist et al., 2003; Hammon et al., 2006), and delayed luteal activity (Wathes et al., 2007), both of which have been shown to be associated with elevated BHBA concentrations.

Excessive NEB has also been linked to milk yield (Duffield et al., 2009). However, defining the level at which elevated NEFA or BHBA are associated with milk production has been difficult because some elevation in these metabolites is characteristic of the transition period and because many other factors affect milk yield. Duffield et al., (2009) described an objective BHBA concentration threshold for the prediction of milk yield early in lactation but the effects of pre- or post-partum NEFA concentrations were not evaluated.

The objectives were to evaluate the association between elevated pre- and post-partum NEFA and BHBA concentrations on reproductive performance and milk production and to establish the metabolite concentrations above which the effects were most likely to occur.

MATERIALS AND METHODS

Study Population and Study Design

A prospective cohort study of progressive dairy herds in the northeastern United States was conducted using a convenience sample of 100 dairy herds. To be included, a herd must have 1) had more than 250 milking cows, 2) had free-stall housing, 3) fed a TMR, and 4) participated in DHIA or used Dairy Comp 305 (Valley Ag Software, Tulare, CA). Herds were excluded from the study if information on herd pregnancy rate was not available and individual animals were excluded from the study if culling data or mature-equivalent 305-d (ME305) milk yield data at 120 DIM were not complete.

In each herd, 2 separate cohorts of animals were sampled on the same day: those 14 to 2 d pre-partum and those 3 to 14 d post-partum. A representative herd sample was obtained by

cross-sectional sampling of approximately 15 apparently healthy animals from each cohort. Sampling 15 animals would give 90% confidence that the sample represents the true within-herd prevalence. Healthy was defined as not being in the sick pen, not currently receiving any medical treatment, and not displaying sick cow signs based on the investigators' interpretation. To reflect common herd demographics, approximately one third of the animals sampled were primiparous.

Ten-milliliter blood samples were collected into plain redtop evacuated tubes from each animal's coccygeal vessel and handled according to recommendations (Stokol and Nydam, 2005). Concentrations of NEFA and BHBA were measured in serum using standardized reagents (NEFA-C, Wako Chemicals USA Inc., Richmond, VA; β-HB, Catachem Inc., Bridgeport, CT) and an automated wet chemistry analyzer (Hitachi 917, Roche Diagnostics, Indianapolis, IN). The sera from the pre-partum cohort were analyzed for NEFA and hemolysis (Stokol and Nydam, 2006). The sera from animals sampled post-partum were analyzed for NEFA, BHBA, and hemolysis (Stokol and Nydam, 2006). Hemolysed samples were discarded.

The reproductive information collected consisted of herd use of ovulation synchronization program and timed AI, voluntary waiting period (VWP), calving date, calving season, DIM at conception, and when applicable, culling date and pregnancy status at culling. Reproductive success was measured as pregnancy within 70 d post-individual herd VWP. This classification was used to standardize the time at risk to get pregnant and to avoid penalizing cows from herds with longer VWP. Pregnancy diagnosis was performed by the herd veterinarian by rectal palpation or ultrasound and recorded by herd personnel. Calving season was dichotomized into 2 categories: warm months (May to September) and cool months (October to April). Parity was dichotomized into 2 groups: parity 1 or ≥2. Body condition score was

determined at time of blood sample collection (Ferguson et al., 1994) and dichotomized into 2 groups: <3.75 or ≥3.75.

Milk production information was collected as ME305 milk yield measured at approximately 120 DIM (incorporating 4 test-days) and was retrieved from DHIA or on-farm Dairy Comp 305 records.

Statistical Analysis

All data was stratified based on time of sample collection, pre- or post-partum. Initially, heifers and cows were evaluated separately, but if the association between the covariates and the outcome of interest was similar they were grouped in the final analyses. Reproduction and milk production were the 2 outcomes of interest and measured as conception within 70 d post-individual herd VWP and ME305 milk yield (kg) assessed at 120 DIM, respectively.

For both outcomes, the metabolites NEFA and BHBA were evaluated as the main categorical predic tors in 3 models: pre-partum NEFA; post-partum NEFA and BHBA; and BHBA alone. Beta-hydroxybutyrate is easily measured in the field; therefore, its effect as a main predictor was modeled separately from NEFA.

Reproduction

Time from individual herd VWP until pregnancy was modeled using a semiparametric proportional hazards model (Cox, 1972) accounting for clustering of cows within herds with Proc Phreg (SAS Institute, 2004). In addition to the main effects of NEFA and BHBA, the covariates BCS, parity, and ME305 milk were evaluated. The ME305 milk data were dichotomized based on the median production of the 2 sampled cohorts. The effect of an elevated metabolite concentration was evaluated by dichotomizing the metabolite concentration within the range of values identified as critical thresholds for prediction of diseases in Ospina et al., (2010). The ranges were as follows: pre-partum NEFA, 0.27 to 0.37 mEq/L; post-partum

NEFA, 0.36 to 0.72 mEq/L; and BHBA, 7 to 12 mg/dL. The dichotomized NEFA and BHBA concentrations that resulted in the smallest chance of committing a type I error and had the largest estimate were kept in the final model.

The Cox proportional hazards models allowed the use of information from animals that did and did not become pregnant; however, animals that were culled before the end of the VWP were excluded from the analysis. Animals not pregnant by the end of the follow- up period were right censored. The proportional hazards assumption was checked statistically by evaluating time-dependent covariates (Allison, 1995). Noninformative censoring was evaluated with sensitivity analysis. Kaplan-Meier estimator graphs (Proc Lifetest) of time to pregnancy within 70 d post-VWP for animals with elevated metabolite levels were created (SAS Institute, 2004).

Milk Production

Mature-equivalent 305-d milk yield was modeled using a mixed effects model (Proc Mixed) with herd as a random effect in SAS (SAS Institute, 2004). In addition to the main predictors (NEFA and BHBA), the covariates BCS, calving season, and when applicable, parity and the interaction between parity and the metabolite concentration were evaluated. The effect of elevated NEFA and BHBA concentrations was evaluated by dichotomizing the concentrations within the range of critical values previously identified. The dichotomized metabolite concentration that resulted in the smallest chance of committing a type I error and largest estimate was kept in the final model.

RESULTS

Descriptive Data

Ninety-one herds were included in the study, and the herd size ranged from 265 to 2,770 with a mean of 827 lactating animals. In total, 2,259 cows were used to evaluate reproductive performance, of which 1,164 were sampled pre-partum (37% heifers and 63% cows) and 1,095

were sampled post-partum (41% heifers and 59% cows). In total, 2,290 animals were used to evaluate milk production, of which 1,183 were sampled pre-partum (36% heifers and 64% cows) and 1,107 were sampled post-partum (41% heifers and 59% cows).

The median ME305 milk production measured at 120 DIM for heifers sampled pre-partum was 12,572 kg and for those sampled post-partum was 12,550 kg. The median milk production for cows sampled pre-partum and post-partum was 12,254 and 12,197 kg, respectively. The VWP ranged from 40 to 75 d. More than 95% of herds used ovulation synchronization programs. The mean pregnancy rate was 19.6 (median 19.5) and ranged from 9 to 32.

Reproduction in the Pre-partum Cohort

Heifers and cows were grouped in the final analyses because the associations between pre-partum NEFA concentrations and reproductive performance were similar. In animals sampled pre-partum, and controlling for BCS, calving season, milk production, and parity, the effect of having a NEFA concentration ≥0.27 mEq/L resulted in 19% decreased risk (hazard ratio = 0.81) of conception within 70 d post-VWP (P = 0.01; Table 3.1). In this model, parity was the only other significant covariate, where animals with parity ≥2 resulted in a 27% decreased risk of conception within 70 d post- VWP (P = 0.001; Table 3.1). Figure 3.1 is a graph of the Kaplan-Meier estimation of animals sampled pre-partum; animals with NEFA values ≥0.27 mEq/L took longer to get pregnant than animals with NEFA values <0.27 mEq/L (P = 0.03).

Reproduction in the Post-partum Cohort

Heifers and cows were grouped in the final analyses because the associations between post-partum NEFA and BHBA concentrations and reproductive performance were similar. In animals sampled post-partum, and controlling for BCS, calving season, milk production, parity, and BHBA dichotomized at 10 mg/dL, the effect of having a NEFA value ≥0.72 mEq/L resulted

in a 16% decreased risk (hazard ratio = 0.84) of conception within 70 d post-VWP (P = 0.05; Table 3.2). In this model, parity was the only other significant covariate where parity ≥2 resulted in a 19% decreased risk of conception within 70 d post-VWP (P = 0.01). When BHBA was evaluated as the only main predictor (i.e., without NEFA in the model) and after controlling for BCS, calving season, and parity, animals with BHBA ≥10 mg/dL had a 13% decreased risk of conception (P = 0.1). In this model, parity was the only significant covariate where parity ≥2 resulted in a 20% decreased risk of conception within 70 d post-VWP (P = 0.01; Table 3.3).

Milk Production in the Pre-partum Cohort

Data from heifers and cows sampled pre-partum were combined in the final analysis because the association between pre-partum NEFA concentrations and milk production were similar for both parity groups. After controlling for parity, BCS, calving season, and the interaction between NEFA and parity, a NEFA concentration ≥0.33 mEq/L in animals sampled pre-partum resulted in a decrease of 683 kg in ME305 milk (P =0.001). Parity was the only other significant covariate (P = 0.01; Table 3.4).

Milk Production in the Post-partum Cohort

The final analyses were stratified by parity because the association between post-partum NEFA and BHBA concentrations and ME305 milk yield were different between heifers and cows. In heifers, after controlling for BCS, calving season, and BHBA dichotomized at 10 mg/dL, heifers with NEFA concentrations ≥0.57 mEq/L had increased ME305 milk yield of 488 kg (P =0.02). There were no other significant covariates in this model (Table 3.5). In heifers sampled post-partum, when BHBA was evaluated as the main predictor, without NEFA in the model, and after controlling for calving season and BCS, heifers with BHBA ≥9 mg/dL had an increase of 403 kg more ME305 milk (P = 0.04; Table 3.6).

In cows sampled post-partum, after controlling for BCS and calving season, those with NEFA ≥0.72 mEq/L had a 647 kg decrease in ME305 milk (P = 0.001; Table 3.7). No other covariates were significant in this model. When BHBA was evaluated as the main predictor, without NEFA in the model, and after con trolling for BCS and calving season, those with BHBA ≥10 mg/dL had a 393 kg decrease in ME305 milk yield (P = 0.04; Table 3.8). No other covariates were significant in this model.

Table 3.1. Cox proportional hazards model for the effect of NEFA, covariates, and cows clustered within herds on d to conception within 70 d post VWP for animals sampled pre-partum (n=1,164)

Variable	Parameter estimate	Standard error	Hazard Ratio	*P*
NEFA[1]	-0.21	0.08	0.81	0.01
BCS[2]	0.02	0.10	1.03	0.8
Season[3]	-0.02	0.09	0.98	0.8
Parity[4]	-0.31	0.09	0.73	0.001
ME 305[5]	-0.03	0.09	0.97	0.6

[1]NEFA: dichotomized < or ≥ 0.27 mEq/L.
[2]BCS: dichotomized at < or ≥ 3.75.
[3]Season: dichotomized into warm (May to September) versus cool months (October to April).
[4]Parity: dichotomized into < or ≥ 2.
[5]ME 305: dichotomized into < or ≥ median for animals sampled pre-partum.

Table 3.2. Cox proportional hazards model for the effect of NEFA, BHBA, covariates, and cows clustered within herds on d to conception within 70 d post VWP for animals sampled postpartum (n=1,095)

Variable	Parameter estimate	Standard error	Hazard Ratio	*P*
NEFA[1]	-0.17	0.09	0.84	0.05
BHBA[2]	-0.07	0.10	0.93	0.4
BCS[3]	0.11	0.08	1.12	0.2
Season[4]	0.12	0.09	1.13	0.2
Parity[5]	-0.21	0.09	0.81	0.01
ME 305[6]	0.06	0.08	1.06	0.5

[1]NEFA dichotomized at < or ≥ 0.72 mEq/L.
[2]BHBA: dichotomized at < or ≥ 10mg/dL.
[3]BCS: dichotomized at < or ≥ 3.75.
[4]Season: dichotomized into two seasons; warm (May to September) versus cool (October to April).
[5]Parity: dichotomized at < or ≥ 2.
[6]ME 305: dichotomized at < or ≥ median for animals sampled postpartum.

Table 3.3. Cox proportional hazards model for the effect of BHBA, covariates and cows clustered within herds on d to conception within 70 d post VWP for animals sampled postpartum (n=1,095)

Variable	Parameter estimate	Standard error	Hazard Ratio	*P*
BHBA[1]	-0.14	0.10	0.87	0.1
BCS[2]	0.09	0.08	1.11	0.3
Season[3]	0.12	0.09	1.13	0.2
Parity[4]	-0.23	0.09	0.80	0.01
ME 305[5]	0.06	0.08	1.07	0.4

[1]BHBA: dichotomized at < or ≥ 10mg/dL.
[2]BCS: dichotomized at < or ≥ 3.75.
[3]Season: dichotomized into two seasons; warm (May to September) versus cool (October to April).
[4]Parity: dichotomized at < or ≥ 2.
[5]ME 305: dichotomized at < or ≥ median for animals sampled postpartum.

Table 3.4. Mixed model for the effect of NEFA, covariates, and herd as a random effect on milk production measured by 120 DIM ME 305 kg for animals sampled pre-partum (n=1,183)

Variable	Difference in ME milk yield (kg)	Standard Error	*P*
NEFA[1]	-683	180	0.0001
BCS[2]	-9	130	0.9
Season[3]	170	220	0.4
Parity[4]	-556	225	0.01
Parity*NEFA[5]	246	270	0.4

[1] NEFA: dichotomized at < or ≥ 0.33mEq/L.
[2] BCS: dichotomized at < or ≥ 3.75.
[3] Season: dichotomized into two seasons; warm (May to September) versus cool (October to April).
[4] Parity: dichotomized at < or ≥ 2.
[5] Interaction between parity and NEFA.

Table 3.5. Mixed model for the effect of NEFA, BHBA, covariates, and herd as a random effect on milk production measured by 120 DIM ME 305 kg for heifers sampled postpartum (n=449)

Variable	Difference in ME milk yield (kg)	Standard Error	*P*
NEFA[1]	488	203	0.02
BHBA[2]	-143	238	0.5
BCS[3]	-0.3	193	0.9
Season[4]	340	273	0.2

[1] NEFA: dichotomized at < or ≥ 0.57mEq/L.
[2] BHBA: dichotomized at < or ≥ 10 mg/dL.
[3] BCS: dichotomized at < or ≥ 3.75.
[4] Season: dichotomized into two seasons; warm (May to September) versus cool (October to April).

Table 3.6. Mixed model for the effect of BHBA, covariates, and herd as a random effect on milk production measured by 120 DIM ME 305 kg for heifers sampled postpartum (n=449)

Variable	Difference in ME milk yield (kg)	Standard Error	*P*
BHBA[1]	403	195	0.04
BCS[2]	38	193	0.8
Season[3]	270	273	0.3

[1] BHBA: dichotomized at < or ≥ 9 mg/dL.
[2] BCS: dichotomized at < or ≥ 3.75.
[3] Season: dichotomized into two seasons; warm (May to September) versus cool (October to April).

Table 3.7. Mixed model for the effect of NEFA, BHBA, covariates, and herd as a random effect on milk production measured by 120 DIM ME 305 kg for cows sampled postpartum (n=658)

Variable	Difference in ME milk yield (kg)	Standard Error	*P*
NEFA[1]	-647	195	0.001
BHBA[2]	-165	205	0.4
BCS[3]	-70	203	0.7
Season[4]	270	242	0.3

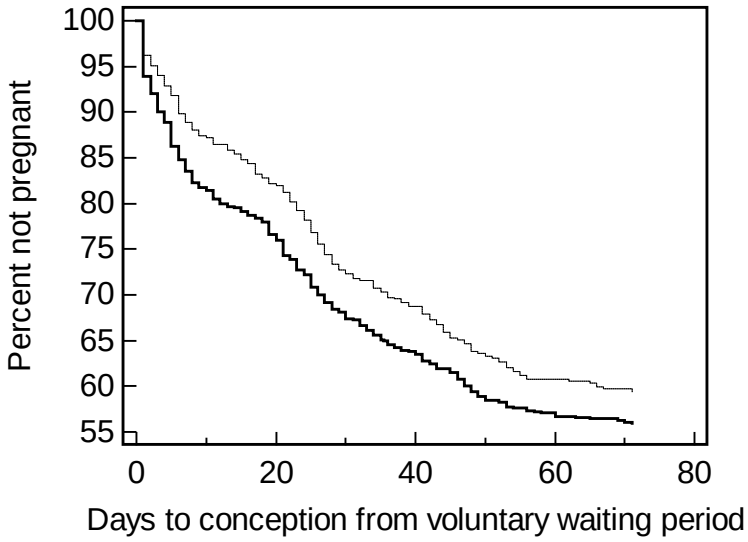
[1] NEFA: dichotomized at < or ≥ 0.72mEq/L.
[2] BHBA: dichotomized at < or ≥ 10 mg/dL.
[3] BCS: dichotomized at < or ≥ 3.75.
[4] Season: dichotomized into two seasons; warm (May to September) versus cool (October to April).

Table 3.8. Mixed model for the effect of BHBA, covariates, and herd as a random effect on milk production measured by 120 DIM ME 305 kg for cows sampled postpartum (n=658)

Variable	Difference in ME milk yield (kg)	Standard Error	*P*
BHBA[1]	-393	195	0.04
BCS[2]	-165	202	0.40
Season[3]	335	247	0.20

[1] BHBA: dichotomized at < or ≥ 10 mg/dL.
[2] BCS: dichotomized at < or ≥ 3.75.
[3] Season: dichotomized into two seasons; warm (May to September) versus cool (October to April).

Figure 3.1. Graph of Kaplan-Meier estimator of time to pregnancy for animals with pre-partum non-esterified fatty acids (NEFA) ≥ 0.27 mEq/L or < 0.27 mEq/L (P = 0.03).



DISCUSSION

Reproduction

Cows with excessive NEB (i.e., concentrations of pre- and post-partum NEFA and BHBA above the thresholds determined in this study) had a decreased risk of pregnancy within 70 d post-VWP (Tables 3.1 to 3.3). This observation is consistent with previous reports that NEB is detrimental to reproductive performance (Villa-Godoy et al., 1988; Wathes et al., 2007). Although elevated concentrations of both NEFA and BHBA resulted in decreased risk of conception, NEFA concentrations generally had a stronger association with reproductive performance than did BHBA. When both post-partum NEFA and BHBA were in the same model, BHBA was not as strong a predictor as NEFA. The strong association between NEFA concentrations and reproductive performance is likely because of the more direct physiological relationship between NEFA concentrations and NEB (Herdt, 2000). The concentration of NEFA increases because of lipolysis, which is positively stimulated by glucagon. Glucagon release is stimulated by hypoglycemia, a direct effect of NEB. Although BHBA concentrations

are also related to NEB, they are regulated by several other factors including NEFA supply to the liver, activity of carnitine palmitoyltransferase (CPT-1), and intramitochondrial activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (Hegardt, 1999; Drackley et al., 2001).

Reproductive performance was evaluated with time to event analysis. Individual herd VWP was incorporated into the time to event analysis to create equal time at risk for pregnancy; thus, avoiding penalizing herds with longer VWP. The VWP could be incorporated as a factor in the time to event analysis because over 95% of the study population used ovulation synchronization protocols and timed artificial insemination. In this population, other commonly used measures of reproductive performance, such as time to first insemination or display of estrus, were not useful indicators because these events were either scheduled or not measured.

Seventy day post-VWP was chosen as a measure of reproductive performance because we wanted to evaluate the effects of elevated NEFA and BHBA concentrations during the transition period on reproduction during this time frame. In addition, conception during this time interval represents an optimal calving interval, such that loss associated with days open may be minimized (Schmidt, 1989; Meadows et al., 2005). A potential limitation of this study was that reproductive performance was not followed until the end of lactation; however, given the scope of this project, extending the follow-up time may have introduced additional confounders and diluted the effect of NEFA and BHBA concentrations.

In addition to NEFA or BHBA concentrations, several other covariates were examined to control for potential confounding: parity, BCS, calving season, and median ME305 milk production. Of these covariates, parity was the only one that was significant in addition to the main effects of NEFA or BHBA. Cows had a decreased risk of pregnancy compared with heifers. This was expected because heifers usually have a higher conception risk than cows. The

effect of BCS on reproductive performance was not significant in this analysis, as has been reported previously (Ruegg and Milton, 1995). However, contrary to other reports (Oseni et al., 2003; Huang et al., 2008), season was not significantly associated with reproductive performance. Interestingly, milk production did not significantly affect reproductive performance (Hansen et al., 1983b; Leroy et al., 2008). Although there has been controversy regarding the association between milk production and reproductive performance, it is not surprising that there was no significant association in the present study. Several studies report that NEB as measured by NEFA or BHBA has a stronger association with reproductive performance than milk production (Reist et al., 2003; Walsh et al.,2007; Wathes et al., 2007). It is important to note that NEFA NEFA concentrations generally increase closer to calving; therefore, studies that sample closer to calving may have higher cut-points associated with downstream outcomes (e.g., milk production, disease events, or re production) than those reported here, which included cows sampled as far as 2 wk pre-partum.

In this study, although information on disease incidence (displaced abomasum, clinical ketosis, metritis, or retained placenta) was available for the animals, it was not included in the investigation of reproductive performance or milk production. The metabolite concentrations used as main predictors in these models are correlated with the development of disease (Ospina et al., 2010) and, as such, would introduce multicollinearity if they were in the same model. Further, the study design employed a cross-sectional sampling strategy such as those typically used in field investigations or in monitoring (Oetzel, 2004). In these scenarios, only information concurrently available would be used to predict reproductive performance or milk production. For example, information on parity and calving season could be incorporated easily, but

information on future development of diseases or serial measurement of NEFA or BHBA concentrations would not.

Milk Production

Milk production was estimated using ME305 milk yield measured at 120 DIM. Mature-equivalent 305-d milk yield was used in this study because it allows us to compare heifers and cows, and, with information from 4 test-days, ME305 is a precise estimate of total milk production (Quist et al. , 2007). Generally, the results of this study are similar to those of other reports; elevated concentrations of BHBA were associated with decreased milk production (Dohoo and Martin, 1984; Duffield et al., 2009). In addition, we also investigated the association of NEFA with milk production and the effect was similar. However, in this study, heifers sampled post- partum showed an inverse relationship; heifers with elevated metabolite levels (NEFA ≥0.57 mEq/L, BHBA ≥9 mg/dL) produced more ME305 milk compared with heifers with concentrations below these levels. Duffield et al., (2009) also found a similar relationship, but did not distinguish between parities. In that study, cows ketotic in wk 2 post-partum had lower first test milk yield, but had higher second and third test milk yields compared with their nonketotic counterparts. Heifers find themselves in a unique physiological circumstance: they have to balance maintenance, growth, and milk production, and as a result may mobilize energy re- sources such as lipid more readily than cows. This concept of homeorhesis may help explain our findings (Bauman and Currie, 1980).

Although elevated concentrations of NEFA and BHBA were significantly associated with ME305 milk production, NEFA concentrations generally had a stronger association with milk production than did BHBA. Pre-partum NEFA concentrations resulted in the lowest chance of committing a type I error (P = 0.0001). When both post-partum NEFA and BHBA were in the same model, NEFA concentrations were so strongly associated with ME305 milk that BHBA

was no longer a significant effect in the model. The strong association between NEFA concentrations and milk production is likely because of the direct physiological relationship between NEFA concentrations and lipid mobilization.

In addition to NEFA and BHBA concentrations, BCS, calving season, parity, and, when applicable, the interaction between parity and the metabolite concentration were examined. In all groups, the single measure of BCS in the transition period was not a significant predictor of milk production. Ruegg and Milton (1995) showed that the change in BCS, and not the single measurement, was predictive of milk production, which may explain our result. Mature-equivalent 305-d milk yield incorporates season into the total calculation; therefore, it was unsurprising that season was not a significant predictor of ME305 milk. Parity was the only significant covariate in animals sampled pre-partum. This is likely because ME305 would be higher for heifers versus cows in progressive herds. It is important to note that the interaction between parity and NEFA concentration was not significant, which means that although there was a difference based on parity, it was not dependent on whether the NEFA concentration was above or below the threshold used in this study.

CONCLUSIONS

The current analysis allowed the opportunity to ex amine the effect of elevated concentrations of pre- and post-partum NEFA and post-partum BHBA on reproduction and milk production at the cow level. The NEFA and BHBA concentrations above which reproduction and production effects were most likely to occur were identified. Generally, compared with animals with metabolite concentrations below the identified thresholds, animals with pre-partum NEFA ≥0.27 mEq/L, post-partum NEFA ≥0.72 mEq/L, and post-partum BHBA ≥10 mg/dL had a decreased risk of pregnancy within 70 d post-VWP. Milk production was decreased in animals with pre-partum NEFA concentrations ≥0.33 mEq/L. In heifers sampled post-partum,

there was an increase in milk production when post-partum NEFA was ≥0.57 mEq/L and BHBA ≥9 mg/dL; however, in cows, milk production was decreased when post-partum NEFA and BHBA were ≥0.72 mEq/L and 10 mg/dL, respectively.

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CHAPTER FOUR

ASSOCIATION BETWEEN THE PROPORTION OF SAMPLED TRANSITION COWS WITH ELEVATED NEFA AND BETA-HYDROXYBUTYRATE (BHBA) AND DISEASE INCIDENCE, PREGNANCY RATE AND MILK PRODUCTION AT THE HERD-LEVEL

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ABSTRACT

The objectives were to: 1) identify the herd-alarm level for excessive negative energy balance, i.e. the proportion of sampled transition cows per herd with elevated pre-partum NEFA, post-partum BHBA and NEFA concentrations that was associated with herd-level incidence of displaced abomasum (DA) or clinical ketosis (CK), pregnancy rate (PR) and milk production and 2) describe the herd-level prevalence of this proportion. This was a prospective cohort study of 60 free-stall, total mixed ration-fed (TMR) herds in the northeast USA. Two animal cohorts were assessed, those 14 to 2 d pre-partum and 3 to 14 d post-partum.Serum was analyzed from approximately 15 animals from each cohort for pre-partum NEFA and post-partum BHBA and NEFA. The herd-level effect of the proportion of animals with concentrations of NEFA or BHBA above a critical threshold was evaluated with a mixed effects model with herd as a random effect. The effect of more than 15% of sampled animals with pre-partum NEFA concentrations ≥ 0.27 mEq/L was associated with a 3.6% increase in DA and CK incidence, 1.2% decrease in PR, and 282 kg decrease in average mature equivalent (ME 305) milk.If more than 15% of animals sampled during the post-partum had BHBA concentrations ≥ 10 to 12 mg/dL, DA and CK incidence increased by 1.8% and PR decreased by 0.8%.ME 305 milk yield was stratified by parity such that if more than 20% of heifers had BHBA concentrations ≥ 12 mg/dL ME 305 decreased by 534 kg and if more than 15% of cows had BHBA concentrations ≥ 10 mg/dL ME 305 milk decreased by 358 kg.If more than 15% of animals sampled had post-partum NEFA concentrations ≥ 0.70 mEq/L then DA and CK incidence increased by 1.7% and if NEFA was ≥ 0.60 mEq/L then PR decreased by 0.9%.ME 305 milk yield results were stratified by parity such that if more than 15% of sampled heifers had post-partum NEFA ≥ 0.60 mEq/L ME 305 milk decreased by 288 kg and if more than 15% of cows had NEFA ≥ 0.70 mEq/L ME 305 milk decreased by 593 kg.The prevalence of herds above the herd alarm level, i.e. herds

having more than 15% of animals sampled with: pre-partum NEFA concentration ≥ 0.30 mEq/L was 75%; BHBA ≥ 12 mg/dL was 40%; and post-partum NEFA ≥ 0.70 mEq/L was 65%.This study showed that if a large enough proportion of cows had elevated metabolite concentrations there were detrimental herd-level effects, and further demonstrates that there is a high prevalence of herds with opportunity for improvement.

Key words: herd alarm level, NEFA, BHBA

INTRODUCTION

The transition from late gestation to early lactation is a critical period in a dairy cow's life because failure to successfully overcome the negative energy balance (**NEB**) caused by the sudden increase in energy demand due to lactation and lagging dry matter intake (Drackley et al., 2001) can increase the risk of detrimental health and reproductive outcomes (Herdt, 2000).At the cow level, elevated beta-hydroxybutyrate (**BHBA**) and non-esterified fatty acid (**NEFA**) concentrations have been used as markers of excessive NEB.Previous studies have shown that elevated concentrations of these metabolites are associated with increased risk of developing detrimental health (Cameron et al., 1998; LeBlanc et al., 2005; Ospina et al., accepted) reproductive (Walsh et al., 2007) and production outcomes (Dohoo and Martin, 1984; Duffield et al., 2009).

Recently, objective cow-level thresholds have been determined for increased BHBA and NEFA concentrations that are associated with disease, reproductive and production outcomes (LeBlanc et al., 2005; Duffield et al., 2009; Ospina et al., accepted).This information allows the identification of individual cows at risk for these downstream outcomes based on their NEB status during the transition period.However, despite all of the NEB information available at the cow-level and its association with downstream outcomes, individual cow strategies for prevention of subclinical disease are still a challenge (Duffield, 2000).This is in part due to the

fact that changes associated with NEB adaptation can start as early as 6 wk pre-partum (Drackley et al., 2001).Efforts to improve NEB status should be implemented at the herd-level where decisions about nutritional management and other aspects of the environment and herd management that in turn affect pre- and post-partum groups of cattle can be addressed appropriately.

Unfortunately, information regarding the appropriate herd alarm levels, i.e. the proportion of sampled animals with elevated concentrations of NEFA and BHBA, have not been well defined (Oetzel, 2004).The objectives of this study were to: 1) identify the herd-alarm level for excessive NEB, i.e. the proportion of sampled animals with elevated NEFA and BHBA that was associated with herd-level changes in the following downstream outcomes: incidence of displaced abomasum (**DA**) and clinical ketosis (**CK**), pregnancy rate (**PR**), and milk production, measured as mature equivalent 305 (**ME 305**) milk and 2) Describe the distribution of the herd-level prevalence of this proportion among herds enrolled in this study.

MATERIALS AND METHODS

Study population and study design

A prospective cohort study was conducted from a convenience sample of dairy herds in New York, Pennsylvania, and Vermont between January 2006 and July 2007; further details of this study can be found in Ospina et. al. (accepted).To be included in this study a herd must have 1) had > 250 milking cows, 2) had free-stall housing, 3) fed a TMR-based diet, 4) participated in DHIA or used Dairy Comp 305 (Version 2009; Valley Ag Software, Tulare, CA) or both, and 5) had follow-up information on at least 10 animals from each cohort.

In each herd, two separate cohorts of approximately 15 healthy, transition animals were sampled cross-sectionally.To reflect common herd demographics, one-third of the animals sampled were primiparous.Animals 14 to 2 d pre-partum were selected to form the pre-partum

cohort and animals 3 to 14 d post-partum were selected to form the post-partum cohort.Healthy was defined as not being in the sick pen, not currently receiving any medical treatment, and not displaying sick cow signs based on the interpretation of the research staff.This cross-sectional sampling scheme was done in order to estimate the herd-level prevalence of animals with an elevated NEFA or BHBA concentration with 90% confidence. 10 ml of blood was collected with a plain red-top evacuated tube from each animal's coccygeal vessel. The samples were handled according to recommendations by Stokol and Nydam (2005).

All samples were analyzed using an automated wet chemistry analyzer (Hitachi 917, Roche Diagnositics, Indianapolis, IN) at the Cornell University Animal Health Diagnostic Center.The sera from the pre-partum cohort were analyzed for NEFA (NEFA-C, Wako Chemicals USA, Inc., Richmond, VA;) and hemolysis while the sera from the post-partum cohort were analyzed for NEFA, BHBA (β-HB, Catachem Inc., Bridgeport, CT) and hemolysis (Stokol and Nydam, 2006).Hemolyzed samples were excluded from the analysis.

Three outcomes of interest were evaluated: incidence of DA or CK in sampled animals, herd PR, and the average ME 305 milk production from sampled animals.Cases of DA or CK within 30 DIM were recorded and for consistency of documentation, disease and case definitions were provided to farm personnel as in Ospina et. al. (accepted).Briefly, DA was defined as movement of the 4^{th} compartment of the stomach to a location on the right (RDA) or left side (LDA) of the cow and detected by auscultating a "ping" sound with finger percussion.Often, this cow had an abrupt decrease in milk production and was off feed.Clinical ketosis was defined as a cow that was off-feed, had sudden weight loss, and decreased milk production, but had no other detectable signs of disease and was treated with dextrose, propylene glycol, and/or steroids (Duffield et al., 1999).The average PR of the two 21 d periods after individual farm voluntary

waiting period (**VWP**) was recorded from Dairy Comp 305 to evaluate early lactation herd reproductive performance.Pregnancy diagnosis was performed by the herd veterinarian by either manual palpation or ultrasound per rectum. ME 305 milk yield was measured at approximately 120 DIM (incorporating 4 test days), and retrieved from DHIA or on-farm Dairy Comp 305 records.

Statistical analysis

In summary, the pre-partum and post-partum cohorts were analyzed separately; however, both cohorts analyzed three models with three continuous.The three outcomes were: 1) incidence of DA or CK or both, 2) herd PR and 3) ME 305 milk.In each model the herd-alarm level for pre-partum NEFA, post-partum BHBA, or post-partum NEFA were evaluated as categorical main effects with herd size as a continuous covariate.Herd size was divided by 100 so that a one unit change in the model was reflective of a change in herd size of 100 animals.Cows and heifers were grouped in the final analyses if the association between the metabolites and the outcome of interest was similar between the two groups.All statistical analyses were performed using SAS v. 9.1 (SAS Institute, Inc., Cary, NC) and evaluated with a mixed effects models (Proc mixed) with herd as a random effect.

Metabolite thresholds and proportion of sampled animals.

The herd-alarm level consists of two numbers: 1) the metabolite (NEFA or BHBA) concentration threshold above which detrimental downstream outcomes are most likely to occur and 2) the proportion of animals with metabolite concentrations above this threshold that is associated with herd-level downstream outcomes. To establish the herd-alarm level both of these parameters were evaluated concurrently. The lowest metabolite concentration and smallest proportion that yielded the smallest chance of committing a type I error and had the largest

change in the outcome of interest (e.g. largest increase in disease incidence) was kept in the final model.

The metabolite concentrations were evaluated within the range identified as critical thresholds associated with individual-cow health effects by previous reports (Ospina et al., accepted; Duffield et al., 2009).According to these reports, the metabolite ranges were: pre-partum NEFA 0.27 to 0.37 mEq/L; post-partum NEFA 0.36 to 0.72mEq/L; and BHBA 7 to 14 mg/dL.

Within each herd, we determined the proportion of animals at or above the thresholds.25% of animals with elevated metabolite levels within each herd the initial proportion used to evaluate the association between the proportion of sampled animals with elevated metabolites and downstream outcomes.In order to increase the sensitivity of the herd-alarm level, the proportion of animals with elevated metabolite levels was lowered by 5% increments.

Prevalence

To identify the prevalence of herds above the herd-alarm levels most commonly associated with downstream outcomes, herds with more than 15% of sampled animals with elevated pre-partum NEFA concentrations ≥ 0.30 mEq/L, BHBA ≥ 12 mg/dL, and post-partum NEFA ≥ 0.70 mEq/L were counted.Bar charts with four categories (≤ 15, >15 to ≤ 25, > 25 to ≤ 35, and > 35) for the proportion of animals with metabolites above the thresholds were created.

RESULTS

Descriptive data

60 herds met the inclusion criteria and from these herds 1672 cows were included in the analysis. In the pre-partum cohort there were 867 animals (37% heifers and 63% cows) and 805 in the post-partum cohort (41% heifers and 59% cows).The number of milking cows per herd

ranged from 353 to 2770 with a mean of 950.The animals sampled had a mean incidence of DA or CK or both of 8.3%, an average herd PR of 19.7% and an average ME 305 milk of 12,487 kg.

Multivariable analysis

Pre-partum cohort- NEFA.

The herd-alarm level for all outcomes (DA or CK, PR, and ME 305 milk) was defined as having more than 15% of animals sampled with pre-partum NEFA ≥ 0.27 mEq/L (Table 4.1). Heifers and cows were grouped in the analysis for all outcomes of interest because the results between the groups were similar.Herds above this alarm level, i.e. when more than 15% of the sampled animals had a pre-partum NEFA concentration ≥ 0.27 mEq/L, had a 3.6% increase in the incidence of DA or CK or both (P = 0.006), a 1.2% decrease in PR (P = 0.006), and a decrease of 285 kg ME 305 milk yield (P = 0.002).Herd size was a significant covariate in the PR and milk production model (P < 0.0001), but not significant in the disease model (P = 0.1).Results indicate that for each additional hundred cows on a farm, PR and ME 305 milk production decreased.

Post-partum cohort- BHBA.

Table 4.2 describes the herd alarm levels associated with post-partum BHBA concentrations and the outcomes of interest. The herd-alarm level for the incidence of DA or CK or both, and PR was defined as having more than 15% of sampled animals with BHBA ≥ 12 mg/dL. Herds above the alarm-level, i.e. when more than 15% of the sampled animals had a BHBA concentration ≥ 12 mg/dL, had a 1.8% increase in the incidence of DA or CK or both (P = 0.03), and a 0.8% decrease in PR (P = 0.03).The results for ME 305 milk were stratified by parity because the herd-alarm level between heifers and cows was different.The ME 305 milk yield herd-alarm level for heifers (n = 335) was defined as having more than 20% of sampled heifers with BHBA ≥ 12 mg/dL, and the herd-alarm level for cows (n = 470) was defined as

having more than 15% of sampled cows with BHBA ≥ 10 mg/dL.Heifers in herds above the alarm-level had a 534 kg decrease in ME 305 milk yield (P = 0.0002), and cows in herds above the alarm-level had a 358 kg decrease in ME 305 milk yield (P = 0.0004).Herd size was a significant predictor (P < 0.0001) of all outcomes except the incidence of DA or CK or both (P = 0.8).Results indicate that for each additional hundred cows on a farm, PR and average ME 305 milk production decreased.

Post-partum cohort- NEFA.

Table 4.3 describes the herd alarm levels associated with post-partum NEFA concentrations and the outcomes of interest. The herd-alarm level for the incidence of DA or CK or both was defined as having more than 15% of sampled animals with post-partum NEFA concentrations ≥ 0.70 mEq/L.Herds above this alarm level had a 1.7% increase incidence of DA or CK or both (P = 0.04).The herd-alarm level for the PR was defined as having more than 15% of animals with NEFA concentrations ≥ 0.60 mEq/L (P = 0.05).Herds above this alarm level had a 0.9% decrease in PR.The results for ME 305 milk were stratified by parity because the herd-alarm level between heifers and cows was different.If more than 15% of the heifers (n = 335) sampled had post-partum NEFA ≥ 0.60 mEq/L then ME 305 decreased by 288 kg (P = 0.07).If more than 15% of the cows (n = 470) sampled had post-partum NEFA concentrations ≥ 0.70 mEq/L then ME 305 decreased by 593 kg (P < 0.0001).Herd size was included as a continuous covariate in all models and was significantly associated (P < 0.0001) with all outcomes except for DA or CK (P = 0.9).Results indicate that for each additional hundred cows on a farm, PR and average ME 305 milk production decreased.

Prevalence

From the 60 herds in the study, the prevalence of herds with more than 15% of sampled animals above the metabolite threshold was: 75% with pre-partum NEFA ≥ 0.30 mEq/L, 40% with BHBA ≥ 12 mg/dL, and 65% with post-partum NEFA ≥ 0.70 mEq/L (Figures 4.1 – 4.3).

Table 4.1. Herd-level effect of elevated pre-partum NEFA concentrations on disease, pregnancy rate and ME 305 milk.

I. Effect on disease[1] if more than 15% of animals sampled with NEFA concentration ≥ 0.27 mEq/L

Variable	Change in percent of disease	Standard error	*P*
NEFA	3.6	1.3	0.006
Herd size[2]	- 0.2	0.1	0.1

II.Effect on pregnancy rate[3] if more than 15% of animals sampled with NEFA concentration ≥ 0.27 mEq/L

Variable	Change in pregnancy rate (%)	Standard error	*P*
NEFA	- 1.2	0.4	0.006
Herd size[2]	- 0.3	0.04	< 0.0001

III. Effect on average ME 305 milk if more than 15% of animals sampled with pre-partum NEFA concentration ≥ 0.27 mEq/L.

Variable	Change in ME 305 milk (kg)	Standard error	*P*
NEFA	- 282	91	0.002
Herd size[2]	- 47	7.2	< 0.0001

[1] Disease is measured as the proportion of sampled animals that developed a displaced abomasum or clinical ketosis or both.
[2] Herd size: 1 unit change = 100 additional cows
[3] Herd pregnancy rate: average of the two 21-day periods post herd voluntary waiting period.

Table 4.2. Herd-level effect of elevated BHBA concentrations on disease, pregnancy rate and ME 305 milk.

I.Effect on disease[1] if more than 15% of animals sampled post-partum with BHBA concentration ≥ 12 mg/dL.

Variable	Change in percent of disease	Standard error	*P*
BHBA	1.8	0.8	0.03
Herd size[2]	0.02	0.08	0.8

II. Effect on herd pregnancy rate[3] if more than 15% of animals sampled post-partum with BHBA concentration ≥ 12 mg/dL on.

Variable	Change in pregnancy rate (%)	Standard error	*P*
BHBA	- 0.8	0.4	0.03
Herd size[2]	- 0.3	0.04	< 0.0001

III. Effect on average ME 305 milk in heifers if more than 20% of heifers sampled post-partum with BHBA concentration ≥ 12 mg/dL.

Variable	Change in ME 305 milk (kg)	Standard error	*P*
BHBA	- 534	141	0.0002
Herd size[2]	- 64	12	< 0.0001

IV. Effect on average ME 305 milk in cows if more than 15% of cows sampled post-partum with BHBA concentration ≥ 10 mg/dL.

Variable	Change in ME 305 milk (kg)	Standard error	P
BHBA	-358	99	0.0004
Herd size[2]	-53	10	< 0.0001

[1] Disease is measured as the proportion of sampled animals that developed displaced abomasum, or clinical ketosis or both.
[2] Herd size: 1 unit change = 100 additional cows
[3] Herd pregnancy rate: average of the two 21-day periods post herd voluntary waiting period.

Table 4.3. Herd-level effect of elevated post-partum NEFA concentrations on disease, pregnancy rate and ME 305 milk.

I.Effect on disease[1] if more than 15% of animals sampled with post-partum NEFA concentration ≥ 0.70 mEq/L

Variable	Change in percent of disease	Standard error	P
NEFA	1.7	0.8	0.04
Herd size[2]	-0.003	0.07	0.9

II. Effect on herd pregnancy rate[3] if more than 15% of animals sampled with post-partum NEFA concentration ≥ 0.60 mEq/L

Variable	Change in pregnancy rate (%)	Standard error	P
NEFA	-0.9	0.5	0.05
Herd size[2]	-0.3	0.04	< 0.0001

III. Effect on average ME 305 milk in heifers if more than 15% of heifers sampled post-partum with NEFA concentration ≥ 0.60 mEq/L

Variable	Change in ME 305 milk (kg)	Standard error	P
NEFA	-288	159	0.07
Herd size[2]	-66	13	< 0.0001

IV. Effect on average ME 305 milk in cows if more than 15% of cows sampled post-partum with NEFA concentration ≥ 0.70 mEq/L

Variable	Change in ME 305 milk (kg)	Standard error	P
NEFA	- 593	107	< 0.0001
Herd size[2]	- 56	10	< 0.0001

[1] Disease is measured as the proportion of sampled animals that developed displaced abomasum or clinical ketosis or both.
[2] Herd size: 1 unit change = 100 additional cows
[3] Herd pregnancy rate: average of the two 21-day periods post herd voluntary waiting period.

Figure 4.1. The prevalence of herds with more than 15% of sampled animals with pre-partum Non-esterified fatty acids (NEFA) concentration ≥ 0.30 mEq/L.

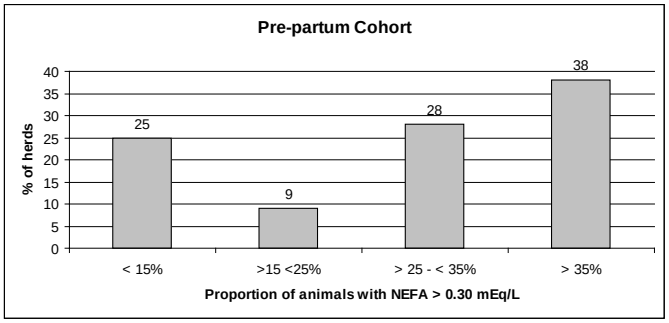


Figure 4.2. The prevalence of herds with more than 15% of sampled animals with beta-hydroxybutyrate (BHBA) concentration ≥ 12 mg/dL.

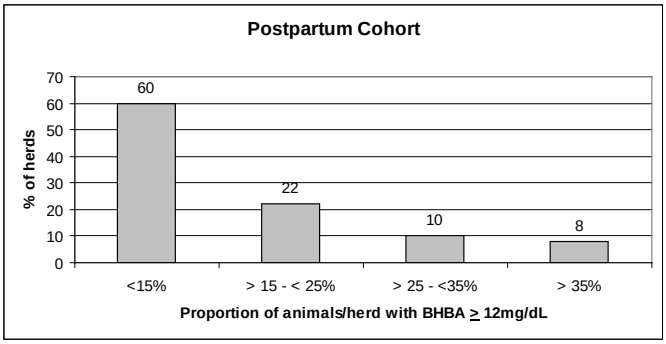
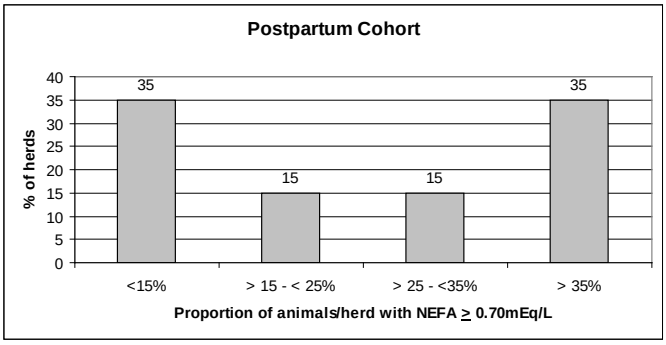


Figure 4.3. The prevalence of herds with more than 15% of sampled animals with post-partum Non-esterified fatty acids (NEFA) concentration ≥ 0.70 mEq/L.



DISCUSSION

Several studies have investigated the association between individual transition animals with elevated NEFA or BHBA concentrations and detrimental downstream outcomes (Cameron et al., 1998; Duffield et al., 2009; Ospina et al., accepted), however herd-level effects of having an excessive proportion of animals with elevated NEFA or BHBA concentrations have not been well defined (Oetzel, 2004).This study defines the herd-alarm level for excessive NEB.The herd-alarm is the proportion of sampled transition cows per herd with elevated pre-partum NEFA, post-partum BHBA and NEFA concentrations that was associated with detrimental herd-level downstream outcomes.The outcomes assessed here were incidence of DA or CK or both, PR and ME 305 milk production.Generally, the results of this herd-level study are in agreement with previous individual cow studies; elevated NEFA or BHBA were associated with detrimental health, reproductive and milk production outcomes (Duffield et al., 2009; Ospina et al., accepted).

The herd-alarm level for excessive NEB, i.e., the proportion of sampled animals with elevated metabolite (NEFA or BHBA) concentrations that were associated with herd-level outcomes was 15%, except when sampling heifers for BHBA with milk production as the outcome where the proportion was 20%. These were the smallest proportions that yielded the largest change in the outcome and the smallest chance of committing a type I error.The smallest proportion was chosen in order to increase the sensitivity of the test i.e. minimize the false negatives.The herd-alarm level for heifers may have been larger than it was for cows due to potential biological differences.Compared to cows, heifers have to balance maintenance, milk production as well as growth.As a result heifers may be more capable of managing mobilized energy resources than cows, thus more tolerant of elevated BHBA concentrations before negative outcomes are measurable.

Within each cohort, the thresholds for metabolite concentrations associated with the outcomes of interest were similar: 0.27 mEq/L for pre-partum NEFA; 10 or 12 mg/dL for BHBA, and 0.60 or 0.70 mEq/L for post-partum NEFA.The small difference in the BHBA concentration associated with effects on milk production is most likely due to a difference in sample size and population; cows and heifers were stratified in the analysis of milk production.As previously mentioned, it is not surprising that heifers would have a higher concentration of BHBA when compared to cows because they need to balance maintenance, milk production in addition to growth and as such may be more capable of using higher sub-clinical levels of ketones before negative effects are seen.The post-partum NEFA concentration was 0.60 mEq/L for both milk production in heifers and PR and 0.70 mEq/L for both milk production in cows and disease.The difference in NEFA concentration between heifers and cows in the milk production model can be explained by the fact that these are different populations.In addition, the smaller sample size of heifers in the milk production model may make it difficult to see a smaller difference between the two groups (herds above the alarm level versus those that are not).Although the probability of saying that there is a difference when there really is no difference in PR is 5%, a post-partum NEFA concentration of 0.60 mEq/L may reflect that the reproductive system may be more sensitive to elevated NEFA concentrations in the transition period than the mechanism that increases the risk for a DA or CK.

To identify whether a herd is above the herd-alarm level and at increased risk for negative downstream outcomes, the proportion of animals with elevated NEFA, BHBA or both can be measured.For herd-level testing a representative sample of adequate size is necessary.It is important to note that the desired confidence in the estimate of the proportion plays a larger role in sample size calculations than herd size.For example, to be 90% confident that the sample is

representative of the herd, with an assumed prevalence of 15% of animals with elevated metabolite concentrations, at least 15 animals at risk would need to be sampled.However, if 12 animals were sampled, in a herd with the same assumed prevalence, the confidence that the sample is representative of the herd is reduced to 75%.

One of the limitations of this study is that in some herds, information on fewer than 15 animals was available.The implication of this is that the confidence that the sample represents the true herd-level prevalence is decreased.In addition, when evaluating the effects on milk production, the groups were stratified by parity because the herd-alarm level for each group was different, further decreasing our sample size per herd. However the herd-alarm level still had a strong association with the outcomes of interest.

All outcomes of interest, incidence of DA or CK, herd PR, and ME 305 milk yield, were analyzed as continuous outcomes.Although pregnancy at the individual cow-level represents a dichotomous outcome, the herd PR can be evaluated as a continuous outcome.At the herd-level PR is represented with a binomial distribution which can be approximated to the normal distribution when the sample size is large (Casella and Berger, 1990).By approximating the binomial distribution to the normal, herd-level PR can be evaluated as a continuous outcome.In addition, to ensure that the cohorts of animals sampled in the transition period were represented in the PR, the animals were followed forward in time and the average of two 21 d periods post individual herd VWP were used.

Herd size was also evaluated as a continuous covariate. The effect of increasing the herd size by 100 cows was significantly associated with PR and ME 305 milk production, but not with DA or CK incidence.In Table 4.3 for example, after controlling for post-partum NEFA concentrations, the effect of increasing herd size by 100 cows did not significantly affect (P =

0.9) disease incidence, but for each additional 100 cows on a farm, PR decreased by 0.3 ($P <$ 0.0001).

Although there has been considerable focus on the study of NEB in the transition period (Drackley, 1999), high producing herds with good reproductive performance such as the ones studied here still had 40% to 75% of herds above the herd-alarm level. This demonstrates that although several herds were capable of benefiting from being below the herd-alarm level, there is still room for improvement.Recognizing herds at risk for increased disease incidence, decreased PR, and decreased milk production based on the effects of elevated NEFA or BHBA concentrations during the transition period may help herds focus on improving energy balance.Herds that choose to focus on herd-level factors for improving negative energy balance at the individual cow level may do so by focusing on nutritional management, diet, comfort, social adaptation and access to feed which may be the best methods of minimizing the lagging DMI during the transition period which is one of the major factors associated with NEB (Drackley et al., 2001).

CONCLUSION

The current study offers herd-level analysis of the transition period based on the identification of both the NEFA and BHBA concentration threshold and the proportion of sampled animals with elevated metabolite thresholds that would mostly likely result in detrimental herd-level outcomes.Compared to herds with a low proportion of sampled animals with elevated NEFA or BHBA concentrations, herds with a high proportion of sampled animals with elevated metabolite concentrations had a higher incidence of DA and CK, lower PR and decreased ME 305 milk production.These herd-level effects were most likely to occur if metabolite concentration thresholds were: pre-partum NEFA $\geq$ 0.27 mEq/L, and post-partum BHBA $\geq$ 10 or 12 mg/dL and NEFA between 0.60 or 0.70 mEq/L.The proportion of animals

with NEFA or BHBA concentrations above the aforementioned thresholds that was associated with herd-level effects ranged from 15 to 20%.These herd-alarm levels may prove useful because generally between 40 to 75% of the herds sampled had more than 15% of animals sampled with NEFA or BHBA concentrations above the thresholds.

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CHAPTER FIVE

TECHNICAL NOTE: THE RISK RATIO, AN ALTERNATIVE TO THE ODDS RATIO FOR ESTIMATING THE ASSOCIATION BETWEEN MULTIPLE RISK FACTORS AND A DICHOTOMOUS OUTCOME

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ABSTRACT

The objectives were (1) to explain why the risk ratio (RR) is an appropriate measure of association when the outcome of interest is dichotomous (e.g., displaced abomasum or no displaced abomasum) in both cohort studies and randomized trials; and (2) to outline an applied method for estimating the RR using currently available software.Interest in the association between multiple risk factors and a yes or no outcome is very common in the dairy industry; historically, logistic regression, which reports odds ratios (OR), was the method available in common statistical packages to evaluate this kind of association.However, the OR can overestimate the magnitude of the response in cohort studies and randomized trials when the outcome frequency is large.In addition, the interpretation of odds is not intuitive; fortunately, recent advances in statistical software have allowed the estimation of the RR.Because SAS software (SAS Institute Inc., Cary, NC) is commonly used to analyze data, this technical note outlines the basic programming code that may be used to estimate the RR from raw data.Example data from a prospective cohort study was used to compare the OR and RR of developing a displaced abomasum or ketosis or metritis based on multiple predictors, their interaction, and a random effect (e.g., herd).

Key words: risk ratio, odds ratio, Poisson, SAS software

TECHNICAL NOTE

Multivariable analysis; that is, evaluating the association, between multiple predictor variables such as main effects, covariates, and potential confounders with an outcome of interest, is common in dairy science research.This kind of analysis with a yes or no outcome (e.g., mastitis or no mastitis) has become more common but estimating the magnitude of the effect has been limited to 2 × 2 tables, ordinary least square (OLS) linear regression, OLS with an arc sine transformation, or logistic regression.

Although odds ratios (OR) can be estimated in any study with a yes or no outcome, risk ratios (RR) can only be estimated in studies where probabilities are measured directly; that is, cohort studies and randomized trials.The calculation of an OR and an RR and the algebraic relationship between them is shown for a general 2 × 2 table in Table A5.1 of the Appendix.Although both measures of association can be estimated this way, when models contain several covariates and interaction terms, this approach is limiting and laborious.

Although OLS linear regression can be used for analysis of data with several covariates, interaction terms, and a dichotomous outcome, several restrictions and assumptions are violated when the outcome is dichotomous (Dohoo et al., 2003) and the probability of success is <0.1 or >0.9 (Cox, 1970).The most severe consequence results from the violation of the homoscedasticity assumption.This violation can lead to inefficient estimates of coefficients and biased standard error estimates, which result in biased test statistics (Allison, 2001).

An alternative approach to multivariable modeling of positive dichotomous outcomes has been logistic regression, which is based on the logit-transformation and maximum likelihood estimation.This transformation replaces the probability of a positive dichotomous outcome with the log odds, and re-establishes the linear link between this and the predictor variables.

A search of the Journal of Dairy Science online through April 6, 2011, revealed that the first article to discuss the use of logistic regression for the analysis of a dichotomous outcome dates to 1987 (Curtis et al.,1987).Since then, 370 articles published in the Journal of Dairy Science have used the exact phrase "logistic regression" in the title, abstract, or text.The OR was frequently used because it is the estimable measure of association in some

study designs (i.e., case-control studies) or because estimating a more appropriate measure of association such as the RR was not readily feasible with commercially available statistical packages.Although the OR and not the RR was estimated, instead of reporting the odds, the results were sometimes misinterpreted as RR and incorrect phrases such as “more likely” or “risk” were used to describe the association between the risk factor(s) and the outcome of interest.

Two problems are associated with estimating the OR in study designs where the RR can be estimated: (1) the OR can overestimate the true effect as the outcome becomes more common, and (2) interpretation of the OR is not intuitive (Holcomb et al., 2001) because, unlike the RR, the OR does not directly measure effects on probability.The objectives of this study are to explain why the RR is the preferred measure of association when the outcome of interest is dichotomous in both cohort studies and randomized trials and to outline an applied method for estimating the RR using SAS version 9.2 (SAS Institute Inc., Cary, NC).

Objective 1: Preference of RR to OR in Certain *Study Designs*

Although estimating the OR is not incorrect, in study designs where the RR can be estimated it is considered the more appropriate measure of association (Greenland, 1987; Spiegelman and Hertzmark, 2005), because it provides estimates of probabilities directly.The OR can overestimate the true effect when interpreted as a probability in studies where the frequency is large, and the interpretation of odds is not as intuitive as probabilities.To demonstrate that the OR can overestimate the RR as the incidence of the outcome increases, simple example calculations of OR versus RR are provided in Table A5.2 of the Appendix.The incidence ranges from 5 to 30%.The example with 30% incidence

could reflect that seen in a study of mastitis and demonstrates the potential overestimation of the effect when an OR is estimated instead of an RR.

The RR can be calculated from cohort studies and cross-sectional studies as well as randomized trials.These are subsets of 2 general study designs, observational and experimental, respectively.When an experiment is not feasible, observational studies are used to evaluate whether an association exists between a risk factor and an outcome of interest.These studies can be divided into 3 groups: cross-sectional, cohort, and case-control.In a cross-sectional study, both exposure and the outcome of interest are determined simultaneously; therefore, information is lacking about whether the exposure preceded the outcome.In a cohort study, subjects are selected based on whether they have been exposed to the risk factor or not and then followed to determine whether they develop the outcome of interest; thus, outcome probabilities can be estimated directly.Conversely, in a case-control study, subjects are selected based on whether they have the outcome of interest (case) or not (control) and then information on whether they were exposed to the risk factor is collected retrospectively.This type of selection is used to investigate rare outcomes; however, it results in a predetermined ratio of cases to controls and only the ratio of the odds can be estimated.

In situations where an experiment is feasible, a randomized trial is considered the best method to determine causality between a risk factor (e.g., treatment) and the outcome of interest.Randomization reduces potential confounding and more control exists over exposure to the risk factor of interest.For example, to determine whether treatment with propylene glycol (exposure) to transition cows reduces the risk of the development of ketosis (outcome), cows can be randomly assigned to receive propylene glycol or placebo, and then

the RR or OR of developing ketosis given administration of propylene glycol can be estimated.

In the preceding example, both the OR and the RR could be estimated.However, the OR reports the ratio of the odds that the cow will develop ketosis, given that she was treated with propylene glycol, to the odds that the cow will develop ketosis, given that she was not treated, whereas the RR reports the ratio of the probability that the cow will develop ketosis, following exposure, to the probability that the cow will develop ketosis when not exposed.Because this was a randomized trial and the RR was estimable, the OR serves only as a potential overapproximation of the RR.

Objective 2: Calculating the RR with Poisson Regression

In light of some of the concerns with the use of the OR in cohort studies (Greenland, 1987) and randomized trials, several methods have been proposed to estimate the RR.The use of a log link with a binary outcome appears to be a good choice because it can calculate the RR directly.However, this method can have issues with convergence because the estimated probabilities are not confined to the allowable parameter space between 0 and 1 (McNutt et al., 2003).Although several techniques to address the convergence issues have been proposed (Zocchetti et al., 1995), they can be complex and difficult to implement.Other authors (Zhang and Yu, 1998; Kleinman and Norton, 2009) advocated the use of logistic regression to estimate the RR through additional calculations, using the fact that the odds is equal to $[P/(1-P)]$, where P = probability, as a way to avoid the parameter space limitations and thus, avoid convergence issues; however, this can lead to biased estimators (McNutt et al., 2003; Zou, 2004).Poisson regression has its own limitations, namely, that it might provide conservative results (Zou, 2004); that is, the confidence interval will be wider.It can have convergence issues due to parameter space

concerns as in the log binomial model, but this is rare in practice and the model will not yield results if it does happen.Poisson regression has several advantages such as, when used with binary data, the estimates are unbiased, which means that Poisson regression will produce consistent estimations of the relative risk (Lumley et al., 2006).Additionally, when used in cohort studies, it can account for different times at risk (Zou, 2004), a function that cannot be supported by the log-binomial model.

The use of Poisson regression to calculate RR has been advocated by several authors (Frome and Checkoway, 1985; McNutt et al., 2003; Zou, 2004).Although Poisson regression is commonly associated with the evaluation of count data, it can also be used for binary data.The only model restrictions are as follows: the dependent variable must be a nonnegative integer and it must have a Poisson distribution conditional on the values of the explanatory variables (Allison, 2001).Data from a prospective cohort study of 1,318 Holsteins in 100 herds (Ospina et al., 2010) were used to demonstrate the method for calculating the RR with PROC GENMOD using SAS.In this study, approximately 15 cows in the postpartum period (3 to 14 DIM) were sampled per herd.The sampling consisted of drawing blood and measuring a single serum BHBA concentration from healthy cows and heifers.These animals were followed to 30 DIM, and within this timeframe, 3 disease outcomes were documented: (1) displaced abomasum, (2) ketosis, or (3) metritis.The dichotomous outcome of interest was the presence or absence of any of the 3 diseases.The predictor variables were (1) exposure to an elevated concentration of BHBA between 3 to 14 DIM (main effect), (2) parity (potential confounder), (3) the interaction between BHBA and parity, and (4) herd as a random effect.The concentration at which BHBA was considered elevated was 10 mg/dL, which was previously determined by receiver operator

characteristic curve analysis (Ospina et al., 2010).Parity was dichotomized as 1 or ≥2. The SAS code without and with the interaction term will be demonstrated and discussed in the next two sections.

Evaluation without interaction term

The RR (without interaction term) can be estimated with PROC GENMOD, as follows:

```
proc genmod descending data = work.BHBAstudy;
class herd BHBA Parity;
model disease = BHBA Parity / link = log dist = Poisson pscale type3;
repeated subject = herd/ type = exch ;
estimate 'BHBA' BHBA 1 -1/ exp;
estimate 'Parity' Parity 1 -1/exp;
run;
```

The first line of the SAS code tells the system what procedure to run, how to read the data, and where to find the data.The procedure is GENMOD and, in this case, the data can be found in a work file titled "BHBAstudy." The word "descending" relates to how the outcome data are read.By default, when evaluating a dichotomous outcome, the program will model the probability of the smallest outcome variable.In most cases, the outcome variable is coded as 0 or 1, where 0 means that the outcome of interest (event) did not occur, and 1 means that the outcome of interest (event) did occur.Research interest usually lies in evaluating the risk of developing the outcome of interest, so adding the "descending" option to the first line tells the program to model the probability of the largest outcome variable.

The second line of the code is the class statement.This tells the program that the variables "herd," "BHBA," and "parity" are categorical variables.Any class variables, including those in the repeated statement should be included in this line.

The third line is the model statement.The model statement defines the equation that will be examined.In this case, the association between the outcome of interest (occurrence of

any of the 3 diseases) and the predictor variables (main effects, exposure to an elevated concentration of BHBA; potential confounders, parity) will be evaluated.To run Poisson regression using PROC GENMOD, the log link and Poisson distribution must be specified after a forward slash (/) in the model statement.

A major assumption of the Poisson distribution is that the variance is equal to the mean (Agresti, 2007); however, unless all factors are controlled for in a model, this is rarely the case and overdispersion usually occurs.Overdispersion does not bias the coefficients, but it can underestimate the standard errors (Allison, 2001).Correcting the standard errors for overdispersion is done by adding the "pscale" option to the model statement.Under the "pscale" option, each standard error is multiplied by the square root of the Pearson chi-squared statistic for testing goodness of fit, and divided by its degrees of freedom.The "pscale" option is preferred to the "dscale" option due to the theory of quasi-likelihood estimation (McCullagh and Nelder, 1989).The "type3" option offers the opportunity to examine the results without regard to the order in which the terms are specified in the model statement and analyses are based on single degree of freedom.Although these score statistics evaluate the same hypothesis as the Z-statistics in the generalized estimating equation (GEE) parameter estimates, the score statistics usually report more conservative P-values (Stokes et al., 2000).Reporting these P-values should be considered in studies with small sample sizes.

The fourth line of code is the repeated statement.In PROC GENMOD, a class variable (e.g., herd) can be used to cluster individual samples (e.g., cows).It is reasonable to suspect that more similarities will exist among cows within the same herd versus those between herds (McDermott et al., 1994a,b).In addition to the measured characteristics

included in the model, many unmeasured characteristics in the herd will likely have an effect on the probability of whether a cow gets any of the 3 diseases, for example.These intra-herd similarities can be taken into account by incorporating the repeated-measures statement and specifying the structure of the correlation matrix as "exch." Choosing the "exch" option specifies a single correlation that applies to any pair of animals within each cluster (Allison,2001), meaning that within each herd, the probability of developing the outcome of interest should be similar given levels of the risk factors, but this probability can be different between herds.In addition, this modification can be used to correctly estimate the standard error for the RR (Zou, 2004).

The fifth and sixth lines of code are the estimate statements.These statements serve 2 functions: allow contrasts and exponentiate the estimate from the base e ("exp" option after the forward slash).This exponentiation facilitates evaluation of the RR because it no longer needs to be done by hand.It is important to examine the estimate statement closely as this will determine the interpretation of the results.The "1 −1" in this statement compares the BHBA concentration ≥10 mg/dL to BHBA <10 mg/dL (the reference level) and estimates the RR with a 95% CI based on the Wald statistic.It is important to note that SAS will choose the predictor variable with the largest numerical value as the reference level.Estimate statements can be written for any variable in the model.The SAS output generated from running this code can be found in Table 5.1.

As shown in Table 5.1, the RR for developing any of the 3 diseases given that BHBA was ≥10 mg/dL was 4.5, meaning that cows with BHBA concentration ≥10 mg/dL were 4.5 times more likely to develop a displaced abomasum, clinical ketosis, or metritis than

cows with BHBA <10 mg/dL.However, the OR estimated using PROC LOGISTIC was a slight overestimation at 5.1.The interpretation of the OR is that a cow with BHBA ≥10 mg/dL has 5.1 greater odds of developing any of the 3 diseases than a cow with BHBA <10 mg/dL.Note that this is not the likelihood or probability of having the event, but specifically the odds.

Evaluation with interaction term

The RR (with interaction term) can be calculated with PROC GENMOD, as follows:

```
proc genmod descending data = work.BHBAstudy;
class herd BHBA Parity ;
model Disease = BHBA Parity BHBA*Parity / link = log dist = Poisson pscale
type3;
repeated subject = herd/ type = exch;
lsmeans BHBA10 * Parity / pdiff cl exp;
run;
```

Evaluating a 1-way interaction is useful for testing the hypothesis that the relationship between one predictor variable and the outcome depends on the level of the second predictor variable.In this example, the interaction between BHBA and parity was evaluated to see if the effect of elevated BHBA on the development of any of the 3 diseases was different if parity = 1 or if parity ≥2, or if the effect of parity on the development of disease was different if BHBA was ≥10 mg/dL or <10 mg/dL.

A few key differences can be seen in this code compared with the code without the interaction term.The first major difference can be found in the third line, the model statement.The model statement now contains an interaction term, "BHBA * Parity." Interaction terms are incorporated into the model by taking the individual variables and adding an asterisk between them; that is, var 1 * var 2 (e.g., BHBA * Parity).Because the BHBA example consisted of categorical variables, a discussion of continuous variables is not

included; however, the interpretation of interactions with categorical variables is similar to that of continuous variables.

The "lsmeans" statement with "pdiff," "cl," and "exp" options is the second major difference in this code.This "lsmeans" statement allows the examination of all the permutations of categorical variables in the interaction terms, and one "lsmeans" statement should be written for each interaction term in the model.The output generated from this statement will give the RR for all of the permutations and the "cl" option provides 95% confidence limits.Although 95% is standard, this can be changed by suggesting a different α; to do so, add "alpha = #" after the "cl" term.This will also work under the "estimate" statement in the previous example.Generally, when an interaction term is included in a model statement there is no longer one single estimate that determines the RR of the outcome based on the terms included in the interaction term; it is now dependent on the level of the second variable.

The SAS output generated from running the model statement can be found in Tables 5.2, 5 . 3, and 5.4.Table 5.2 contains the information from running the model statement, the output from the LSMEANS statement is in Table 5.3, and the output from the PDIFF, CL, and exp options are found in Table 5.4.The inclusion of the interaction term in the model statement means that the single estimate listed in Table 5.2 of 1.28 for BHBA can no longer solely be used to estimate the RR of developing any of the 3 diseases, because the effect of BHBA on disease risk depends on the level of parity.Also, in Table 5.2, the estimate of 0.60 for the interaction term (BHBA * Parity) represents the change in the estimate of the main effect (BHBA) when the other variable (parity) changes

by 1 level.The P-value associated with the interaction term at this level of analysis is evaluating whether the interaction has a statistically significant effect on the outcome.

The information in Table 5.3 offers the opportunity to examine the risks of developing the outcome (any of the 3 diseases).See Table A5.3 in the Appendix for the mathematical formulation used to evaluate the interaction term.To calculate the RR, the information in Table 5.4 or the calculations in the Appendix are necessary.The information in Table A5.3 shows how the numbers in Table 5.3 were estimated; that is, where they came from, and shows simple calculations of the RR when an interaction term is included.Table 5 . 4 allows independent evaluation of each of the permutations of the interactions and tests whether the difference is significantly different from zero.The column labeled "exponentiated" has the RR for each of the permutations of the interaction term.The confidence limits for the RR are estimated in Table 5.4.The Appendix has sample calculations of RR of interest.Because a log link was used, the natural log is taken on both sides of the equation to evaluate the estimates.In this example, the RR for the development of disease given that BHBA was ≥ 10 mg/dL was 6.6 when parity was =1, and 3.6 when parity was ≥ 2.

Because incorporation of an interaction term can make interpretation of the model more complex, the decision to include an interaction term in a model should not be taken lightly.As previously stated, Table 5.4 allows the evaluation of all permutations of the interaction model and in this case, no significant difference (P = 0.17, 0.27) was found when permutations were compared across parity levels and BHBA levels were held constant.This information along with the large likelihood that the difference based on parity was due to chance (P = 0.27) may result in deciding to remove parity and the interaction term from the model.

Poisson regression was used to estimate the RR.Poisson regression analysis is appropriate when the response variable is a count or a rate and the results can be interpreted as a rate ratio (Frome and Checkoway, 1985).Counts represent the number of events that occurred over an observed period; however, if that period is similar between all subjects, then the rate ratio approximates the RR.This relationship can be seen with the following equations of a hypothetical example when each cow contributes 1 cow-week of time at risk:

$$Rate\ ratio = \frac{Incidence\ rate\ in\ the\ exposed}{Incidence\ rate\ in\ the\ unexposed} = \frac{3\ cases/10\ cow-weeks}{1\ case/10\ cow-weeks}$$

$$Risk\ ratio = \frac{Incidence\ risk\ in\ the\ exposed}{Incidence\ risk\ in\ the\ unexposed} = \frac{3\ cases/10\ exposed\ cows}{1\ case/10\ exposed\ cows}$$

It should be noted that if some counts correspond to different periods at risk than others, then the rate at which the events occur must be included in the model (Stokes et al., 2000; Zou, 2004).This is done by including an "offset" term after the forward slash in the model statement (e.g., offset = time at risk).The natural log of the time at risk should be calculated to include this information in the model.In the current BHBA study, all animals were at risk for similar periods, so the offset term was not included (i.e., was equal to 1).

Interpretation of an RR is intuitive.Many studies that estimate an OR interpret it incorrectly as an RR (Holcomb et al., 2001).It is important to note that when the incidence of the outcome is low (e.g., ≤ 5%), the RR and OR are very similar in magnitude.Yet, the difference between the estimate of the OR versus the RR becomes larger and therefore more of a concern as the incidence becomes greater (Appendix Tables A5.1 and A5.2).The RR reports the likelihood

that the outcome of interest will happen, given a certain level of the risk factor, and is based on the idea that the sample for this calculation is representative of the true population.This SAS code will estimate the RR and therefore, eliminates the need to estimate the OR in cohort studies and randomized trials.

Table 5.1. SAS output for estimating the risk ratio (RR) of developing a displaced abomasum, ketosis, or metritis with Poisson regression using PROC GENMOD.

Analysis Of GEE Parameter Estimates

Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		-3.0450	0.2097	-3.4560	-2.6341	-14.52	<0.0001
BHBA[1]	1	1.4946	0.1828	1.1363	1.8529	8.18	<0.0001
BHBA	0	0.0000	0.0000	0.0000	0.0000	.	.
Parity[2]	1	0.0350	0.1738	-0.3057	0.3757	0.2	0.8406
Parity	2	0.0000	0.0000	0.0000	0.0000	.	.

Score Statistics for Type 3 GEE analysis

Source	DF	Chi-Square	Pr > ChiSq
BHBA	1	29.32	<0.0001
Parity	1	0.04	0.8437

Contrast Estimate Results

Label	Estimate	Standard Error	Alpha	95% Confidence Limits		Chi-Square	Pr>ChiSq
BHBA	1.4946	0.1828	0.05	1.1363	1.8529	66.84	<0.0001
Exp(BHBA)	4.4576[3]	0.8149	0.05	3.1152	6.3785		
PARITY	0.0350	0.1738	0.05	-0.3057	0.3757	0.04	0.8406
Exp (Parity)	1.0356[4]	0.1800	0.05	0.7366	1.4560		

[1] β-hydroxybutyrate (BHBA) concentration dichotomized: 10 if < 10 mg/dL (reference level) and 1 if ≥ 10 mg/dL.
[2] Parity dichotomized: 1 if parity =1 and 2 if ≥ 2 (reference level).
[3] Risk ratio of developing any of the three diseases if BHBA concentration was ≥ 10 mg/dL.
[4] Risk ratio of developing any of the three diseases if Parity was =1.

Table 5.2. Output from SAS software (SAS Institute Inc., Cary, NC) for estimating the risk ratio (RR) with Poisson regression using PROC GENMOD to evaluate the model[1] with an interaction term of BHBA and Parity.

Analysis of GEE Parameter Estimates

		Empirical Standard error Estimates			
Parameter	Estimate	Standard Error	95 % Confidence Limits		Pr > |Z|
Intercept	-2.9157	0.2092	-3.3257	-2.5057	<0.0001
BHBA[2]	1.2844	0.2072	0.8783	1.6905	<0.0001
Parity[3]	-0.3265	0.2953	-0.9053	0.2523	0.2689
BHBA * Parity	0.6008	0.3447	-0.0749	1.2765	0.0814

[1] The model: disease outcomes (displaced abomasum, or ketosis, or metritis) = BHBA[2] + Parity[3] + BHBA * Parity + cows clustered within herds

[2] β-hydroxybutyrate (BHBA) concentration dichotomized: 10 if < 10 mg/dL (reference level) and 1 if ≥ 10 mg/dL

[3] Parity dichotomized: 1 if parity =1 and 2 if ≥ 2 (reference level).

Table 5.3. Abbreviated SAS software (SAS Institute Inc., Cary, NC) output generated from LSMEANS statement[1].

BHBA * Parity Least Square Means

BHBA[1]	Parity[2]	Estimate	Standard Error	Z value	Pr > |Z|
1	1	-1.3569	0.1658	-8.18	<0.0001
1	2	-1.6313	0.1501	-10.86	<0.0001
0	1	-3.2422	0.2672	-12.14	<0.0001
0	2	-2.9157	0.2092	-13.94	<0.0001

[1] The following columns were omitted: alpha, lower and upper confidence limits for estimate, exponentiated estimate, and 95% CL of exponentiated estimate.

Table 5.4. Abbreviated SAS software (SAS Institute Inc., Cary, NC) output obtained from the 'PDIFF CL Exp' options in the LSMEANS statement[1].

Differences of Least Square Means

BHBA	Parity	BHBA	Parity	Estimate	Standard Error	*P*[2]	Exponen.[3] lower	Exponen.[4] upper	Exponentiated[5]
1	1	1	2	0.2743	0.1979	0.1658	0.8926	1.9392	1.3156
1	1	0	1	1.8852	0.3009	<0.0001	3.6526	11.8817	6.5878
1	1	0	2	1.5587	0.2418	<0.0001	2.9588	7.6348	4.7529
1	2	0	1	1.6109	0.2879	<0.0001	2.8514	8.7932	5.0073
1	2	0	2	1.2844	0.2072	<0.0001	2.4069	5.4223	3.6126
0	1	0	2	-0.3265	0.2953	0.2689	0.4044	1.2870	0.7215

[1] The following columns were omitted: Z value, alpha, lower and upper confidence limits for estimate.

[2] P-value: for the hypothesis that the difference between the two levels within the interaction is different from zero.

[3] 95% confidence limits of exponentiated estimate, i.e., 95% Confidence limits for risk ratio for specific interaction.

[4] The risk ratio for each permutation of the interaction term.

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APPENDIX

In a case control study (based on the 2 X 2 table in Table A1), the odds ratio (OR) is calculated as follows:

$$Odds\ Ratio = OR = in\ a\ case\ control\ study(based\ on\ 2\ by\ 2\ table\ above)$$
$$= \frac{odds\ that\ a\ case\ was\ exposed}{odds\ that\ a\ control\ was\ exposed} = \frac{a/b}{c/d} = \frac{ad}{bc}$$

Table A5.1. An example of a 2 x 2 table.

	Outcome (Positive)[1]	**Outcome (Negative)[2]**	**Total**
Exposed to Risk factor	a	b	a + b
Not- exposed to Risk factor	c	d	c + d
Total	a + c	b + d	a+b+c+d

[1] Positive outcome = individual developed the outcome of interest
[2] Negative outcome = individual did not develop the outcome of interest

In a cohort study or randomized trial, the risk ratio (RR) is calculated as follows:

$$Risk\ Ratio = RR = in\ a\ cohort\ study\ or\ randomized\ trial$$

$$= \frac{incidence\ of\ outcome\ in\ those\ exposed\ to\ risk\ factor}{incidence\ of\ outcome\ in\ those\ not\ exposed\ to\ risk\ factor} = \frac{\frac{a}{a+b}}{\frac{c}{c+d}}$$

Thus ($when\ a\ and\ c\ are\ relatively\ small$), the RR approximates the OR as follows:

$$= \frac{\frac{a}{a+b}}{\frac{c}{c+d}} = \frac{a\ (c+d)}{c\ (a+b)} \sim \frac{ad}{bc}$$

Table A5.2. Five Sample calculations of OR and RR[1]

Example 1. Incidence of the outcome was 2.5% and χ^2 *P-value* testing a difference between the exposed and not exposed group was 0.002.The OR and RR were similar (4.1 vs. 4, respectively).

Incidence 2.5%	Outcome (+)	Outcome (-)	Total	Results	
Exposed (+)	20	480	500	**OR**	4.1
Exposed (-)	5	495	500	**RR**	4.0
Total	25	975	1000	χ^2	$P = 0.002$

Example 2. Incidence of the outcome was 5% and χ^2 *P-value* testing a difference between the exposed and not exposed group was < 0.001.The OR was larger than RR (4.3 vs. 4, respectively).

Incidence 5%	Outcome (+)	Outcome (-)	Total	Results	
Exposed (+)	40	460	500	**OR**	4.3
Exposed (-)	10	490	500	**RR**	4.0
Total	50	950	1000	χ^2	$P < 0.001$

Example 3. Incidence of outcome was 10% and χ^2 *P-value* testing a difference between the exposed and not exposed group was < 0.001.The OR was larger than RR (4.6 vs. 4, respectively).

Incidence 10%	Outcome (+)	Outcome (-)	Total	Results	
Exposed (+)	80	420	500	**OR**	4.6
Exposed (-)	20	480	500	**RR**	4.0
Total	100	900	1000	χ^2	$P < 0.001$

Example 4. Incidence of outcome was 15 % and χ^2 *P-value* testing a difference between the exposed and not exposed group was < 0.001.The OR was larger than RR (5 vs. 4, respectively).

Incidence 15%	Outcome (+)	Outcome (-)	Total	Results	
Exposed (+)	120	380	500	**OR**	5.0
Exposed (-)	30	470	500	**RR**	4.0
Total	150	850	1000	χ^2	$P < 0.001$

Example 5. Incidence of outcome was 30 % and χ^2 *P-value* testing a difference between the exposed and not exposed group was < 0.001.The OR was larger than RR (6.8 vs. 4, respectively).

Incidence 30%	Outcome (+)	Outcome (-)	Total	Results	
Exposed (+)	240	260	500	**OR**	6.8
Exposed (-)	60	440	500	**RR**	4
Total	300	700	1000	χ^2	$P < 0.001$

[1] Examples have outcome incidences that range from 2.5 to 30%.This is used to illustrate the point that the discrepancy between OR and RR gets larger as the incidence increases.

Table A5.3. Calculations of the risk of developing any of the 3 diseases and example calculations of RR with the incorporation of an interaction term.

Model: $Y^{1} = B_0 + B_1 * BHBA^{2}(0,1) + B_2 * Parity^{3}(0,1) + B_3{}^{4} * BHBA(0,1) * Parity(0,1)$

Y=	B_0	B_1 * BHBA	B_2 * Parity	$B_3{}^{4}$ * BHBA*Parity	Risk of Y^{5}	Given
DA, CK or MET	- 2.9	+ 1.3 * 1	- 0.33 * 1	+0.60 * 1 * 1	$e^{-1.3}$	BHBA ≥ 10mg/dL Parity = 1
	- 2.9	+ 1.3 *1	- 0.33 * 0	+0.60 * 1 * 0	$e^{-1.6}$	BHBA ≥ 10mg/dL Parity ≥ 2
	- 2.9	+ 1.3 * 0	- 0.33 * 1	+0.60 * 0 * 1	$e^{-3.2}$	BHBA < 10mg/dL Parity = 1
	- 2.9	+ 1.3 * 0	- 0.33 * 0	+0.60 * 0 * 0	$e^{-2.9}$	BHBA < 10mg/dL Parity ≥ 2

[1] Y= any combination of the three diseases = displaced abomasum (DA), clinical ketosis (CK), metritis (MET).
[2] β-hydroxybutyrate (BHBA) concentration dichotomized: 0 if < 10mg/dL (reference level) and 1 if ≥ 10 mg/dL.
[3] Parity dichotomized: 1 if parity =1 and 0 if ≥ 2 (reference level).
[4] $B_3 = 0.6$, this is the change in the estimate of the main effect (BHBA) when the other variable (parity) changes by one level.
[5] The exponent calculated here, is the estimate in the column 'estimate' in Table 2A.

Based on the information in Table A5.3, the following are examples of how the risk ratio (reported as "Exponentiated" in Table 5.4) were derived:

Sample calculations of risk ratios.

		Risk Ratio	
$= \frac{e^{\cancel{B0}+B1(1)+\cancel{B2(1)}+B3\,(1*1)}}{e^{\cancel{B0}+B1(0)+\cancel{B2(1)}+B3(0*1)}}$	$= \frac{e^{B1(1)+B3(1)}}{e^{0+0}}$ $= \frac{e^{1.28+0.6}}{e^{0}=1}$	$e^{1.88}$ =6.6	The risk ratio of developing any of the 3 diseases when BHBA was ≥ 10mg/dL and parity =1.
$= \frac{e^{\cancel{B0}+B1(1)+\cancel{B2(0)}+B3\,(1*0)}}{e^{\cancel{B0}+B1(0)+\cancel{B2(0)}+B3(0*0)}}$	$= \frac{e^{B1(1)+0+0}}{e^{0+0+0}}$ $= \frac{e^{1.28}}{e^{0}=1}$	$e^{1.28}$ =3.6	The risk ratio of developing any of the 3 diseases when BHBA was ≥ 10mg/dL and parity ≥ 2.

CHAPTER SIX

EVALUATION OF POOLED VERSUS INDIVIDUAL ANIMAL SAMPLES AND MULTIPLE COW-SIDE TESTS USED TO MONITOR HERDS FOR EXCESSIVE NEGATIVE ENERGY BALANCE

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ABSTRACT

The objectives of this study were to compare the negative energy balance (**NEB**) status of a herd based on individual samples versus pooled samples using beta-hydroxybutyrate (**BHBA**) concentrations; to estimate the herd-level sensitivity (**HSe**) and herd-level specificity (**HSp**) based on simulated individual animal sampling (varying sample size, underlying herd-level prevalence of elevated BHBA, and test sensitivity and specificity); and to estimate the herd predictive value positive (**HPV+**) and herd predictive value negative (**HPV-**) under the same simulation.

Based on previous reports, the concentration of BHBA above which animals were considered at risk for excessive NEB was ≥ 12mg/dL and if more than 15% of the animals tested per herd had BHBA concentrations above this, then the herd was considered at increased risk.

Twelve animal samples from 12 herds were evaluated both individually and the samples were pooled to compare the sensitivity and specificity of a pooled herd sample versus analyzing the proportion of individual samples which had BHBA concentrations ≥ 12 mg/dL.The kappa statistic was 0.13 (95% Confidence Interval **CI**: -0.08 to 0.33), McNemar's p-value was 0.008 and sensitivity and specificity of the pooled samples were 30 (95% CI: 7 to 65%) and 100 (95% CI: 19 to 100), respectively.

Using a test with 0.96 sensitivity and 0.98 specificity, while varying the underlying within-herd true prevalence from 0 to 40% and the between-herd true prevalence also from 0 to 40%, the HSe, HSp, HPV+, and HPV- values were estimated.Additionally, four different tests with varying sensitivities (96, 90, 88, and 78) and specificities (98, 86, 90, 96) were compared in two simulations, one with the underlying within and between herd true prevalence at 20% and the second with these prevalence parameters at 40%.

Pooled samples had a very low sensitivity, thus a large number of false negatives thereby increasing the potential for missing opportunities for improvement.It is recommended to evaluate samples individually by analyzing the proportion of animals with elevated BHBA concentrations. Although the underlying true prevalence of the condition being evaluated will affect the herd-level parameters, it is recommended to test 15 animals to maximize the HPV- and 20 animals to maximize the HPV+.If more than 15% (≥ 2/15 or ≥3/20) of the animals sampled had BHBA ≥ 12 mg/dL, the herd is considered at risk for the negative outcomes associated with excessive NEB.

Key words: herd alarm level, pooling test, BHBA

INTRODUCTION

Excessive negative energy balance (**NEB**), identified by increased levels of circulating non-esterified fatty acids (**NEFA**) and β-hydroxybutyrate (**BHBA**), during the transition period has been associated with increased disease (Ospina et al., 2010a) and decreased reproductive and milk production performance at both the individual animal (Duffield et al., 2009; Ospina et al., 2010c; Chapinal et al., 2011) and herd-level (Ospina et al., 2010b).Although much information has been gained from research focused on the effects of excessive NEB on individual animals; most management strategies intended to correct NEB are best implemented at the group or herd-level.The ability to classify a herd as having excessive NEB, i.e., above the herd alarm level, can help detect opportunities for improvement.

A recent study (Ospina et al., 2010b) identified the herd alarm level as having more than 15% of sampled transition animals with BHBA concentrations ≥ 12 mg/dL.Herds above this alarm level had higher displaced abomasa (**DA)** and clinical ketosis (**CK**) incidence, decreased pregnancy rate (**PR**), and decreased milk production.The accuracy of this herd-based test depends primarily on the underlying true prevalence of the disease being monitored, individual

test sensitivity and individual test specificity; however, herd-level parameters such as herd-level sensitivity (**HSe**), herd-level specificity (**HSp**), herd-level predictive value positive (**HPV+**), and herd-level predictive value negative (**HPV-**) should also be evaluated. The HSe is the probability of having a herd test positive when it really does have excessive NEB, while the HSp is the probability of having a herd test negative when it really does not have excessive NEB. The HPV+ estimates the probability that a positive test came from a positive herd, conversely, the HPV – determines the probability that a negative test came from a negative herd.

In order to correctly evaluate NEB at the herd-level, appropriate sample collection is critical, but it may be difficult to decide when to sample cows, how many cows to sample, and whether it is possible to pool the samples.These questions are of particular importance because the test results will be used to monitor progress and consequently affect management decisions at the herd-level.The objectives of this study are to: 1) to compare the NEB status of a herd based on individual samples versus pooled samples using BHBA concentrations from data collected from Ospina et al., (2010a), 2) estimate the HSe and HSp based on simulated individual animal sampling (varying sample size, underlying herd-level prevalence of elevated BHBA, and test sensitivity and specificity), and 3) estimate the HPV+ and HPV- under the same simulation. Because larger farms (>600 milking cows) will have animals at risk on a weekly basis, the frequency of sampling will also be discussed.

MATERIALS AND METHODS

Pooled samples

Herds from a prospective cohort study (Ospina et al., 2010a) with a minimum of 12 samples in the post-partum cohort were used in the analysis which compared individual versus pooled samples.During the study, herds were visited once and blood was sampled from 12 to 15 healthy animals 3 to 14 DIM.Serum samples from this study were collected, handled and stored

according to sample handling recommendations (Stokol and Nydam, 2005; Stokol and Nydam, 2006).The sera were analyzed for NEFA (NEFA-C, Wako Chemicals USA, Inc., Richmond, VA) and BHBA (β-HB, Catachem Inc., Bridegeport, CT) at the Cornell University Animal Health Diagnostic Center.

To ensure that the herds included in the pooling analysis represented the range of metabolite concentrations, the eligible herds were stratified into quartiles based on mean post-partum NEFA concentration.The arithmetic means and quartiles of the post-partum NEFA concentrations were calculated using commercial software (SAS v. 9.1, SAS Institute Inc., Cary, NC).The quartiles for post-partum NEFA were: < 0.42 mEq/L; ≥ 0.42 and < 0.57 mEq/L; ≥ 0.57 and < 0.73 mEq/L; and ≥ 0.73 mEq/L.Three herds with post-partum NEFA means within each quartile were randomly selected with a ranuni seed for the analysis and resulted in a total of 12 herds.From these 12 herds, 12 individual animal samples were also randomly selected using simple random sampling without replacement. The selected individual serum samples were thawed at room temperature for four hours and pooled herd samples were created with 200 μL aliquots from each individual sample.

Based on previous reports (Ospina et al., 2010b), herds were defined as having excessive NEB if the proportion of individual samples with a metabolite concentration above the threshold was more than 15 % (≥ 2 animals from 12).The metabolite threshold for BHBA was ≥12 mg/dL.To assess whether a herd had excessive NEB based on the pooled sample, the concentration of the pooled sample was compared to the metabolite threshold of ≥12 mg/dL.Herds with pooled sample concentrations ≥12 mg/dL were considered to have excessive NEB.

McNemar's p-value, Kappa statistic, sensitivity and specificity analysis were done to compare between individual and the pooled tests using SAS v. 9.2 (SAS Institute, Inc., Cary, NC).McNemar's p-value evaluates bias between the two proportions and the Kappa statistic evaluates the agreement (beyond chance) between the two tests (Dohoo et al., 2003a; Dohoo et al., 2003b).Sensitivity and specificity analysis compared the pooled test to the individual tests and were calculated using Med Calc version 9.5.2.0 (Schoonjans, 2008).

Individual tests- simulation analysis

At the herd-level, the HSe and HSp are affected by the true prevalence (**TP**) of the condition of interest, the number of animals tested, the critical threshold used to decide whether the herd is positive (herd alarm level), and the sensitivity and specificity of the test used.The sensitivity and specificity used for this part of the analysis were: 0.96 and 0.98, respectively.However, given that there are various tests used to measure ketones each with differing sensitivities and specificities, the effect on HSe, HSp, HPV+ and HPV- were evaluated given the following test sensitivities and specificities: 0.96 and 0.98; 0.90 and 0.86; 0.88 and 0.90; and 0.78 and 0.96 while keeping the within and between herd true prevalence at 20% and repeating the analysis with these prevalence parameters at 40%.

Formulas presented by Martin et al., (1992) were used to estimate the HSe and HSp given: various permutations of different levels of TP (0 to 40%) and number of animals tested (10 to 25), all based on a 15% positive critical threshold.These calculations were done assuming no sampling variability, i.e., the sample prevalence is equivalent to the herd prevalence.

The HSe is the probability of getting equal to or more than the critical threshold number of animals expected with BHBA concentrations ≥ 12 mg/dL, given that the herd is truly positive.The critical threshold used to determine whether a herd was positive was 15% or as close as possible considering that animals are whole numbers, so for example when the sample

size was 10, 15, 20 or 25, the cut-points were 2, 2, 3, and 4, respectively.The HSp is the probability of getting no positive tests in a herd with 0 true prevalence of animals with BHBA ≥ 12 mg/dL.

The herd-level predictive value positive (HPV+) and herd-level predictive value negative (HPV-) were estimated using the standard formulae for predictive values (Martin et al., 1992; Ospina et al., 2010b).The HPV + determines the probability that a positive herd test actually comes from a positive herd.The HPV – determines the probability that a negative herd test actually comes from a negative herd. These values are calculated based on HSe, HSp, and the true prevalence of infected herds; this is the pre-test probability that a herd is positive.The underlying TP of infected herds is estimated to be around 40% based on work presented by (Ospina et al., 2010b), however, the HPV+ and HPV- were evaluated on a range starting with a conservative estimation of 10% and up to 40% pre-test probability of being positive.

RESULTS

Pooled samples

The proportion of the twelve herds with excessive NEB based on individual BHBA tests was 0.8, and the proportion of herds with excessive NEB based on pooled BHBA samples was 0.3.The McNemar's p-value was 0.008 and the kappa statistics was 0.13 (95% CI: -0.08 to 0.33).The sensitivity and specificity of the pooled BHBA test when compared to the individual tests was 30 (95% CI: 7 to 65%) and 100 (95% CI: 19 to 100), respectively.

Individual tests- simulation analysis

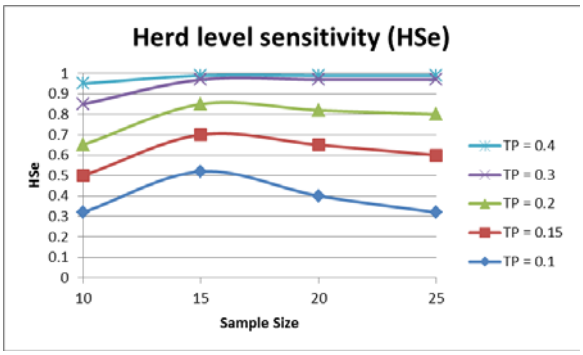
In the simulation study, when the test sensitivity and specificity were held constant at 0.96 and 0.98, respectively, the HSe varied based on underlying true prevalence and sample size (Figure 6.1).Although HSe increases as within herd TP increases, it is at its highest when the sample size is 15. This is because HSe decreases as the number of samples required to call a herd

positive increases.The HSp is based on the assumption that the herd is negative; therefore the underlying TP does not affect the HSp, however, HSp increases as the number of tests required to call a herd positive increases.For the following sample sizes: 10, 15, 20, and 25; the HSp was .98, .97, .99, and .99, respectively (Figure 6.1).

The HPV+ and HPV- (Figure 6.2) depend on both true within herd prevalence of elevated BHBA and the between herd-level prevalence of infected herds, i.e., the pre-test probability of a herd being positive.HPV+ increases as the elevated BHBA prevalence within herd and between herds increase.Although HPV+ generally increases as sample size increases, there is a notable drop when the sample size is 15.The HPV- increases as the within herd true prevalence increases, but there is a slight decrease as the prevalence of positive herds increases and there is a notable increase when the sample size is 15.

Figure 6.3 demonstrates the effect of changing test sensitivity and test specificity on HSe and HSp, and Figure 6.4 demonstrates the effect of these changes on HPV+ and HPV-.Although the herd-level parameters are different, the patterns seen previously when the test sensitivity was 0.96 and test specificity was 0.98 are similar.

Figure 6.1. Herd-level sensitivity (HSe) and herd-level specificity (HSp) based on different sample sizes (10 to 25) and varying true prevalence (0 to 40%) of animals with BHBA concentrations ≥12 mg/dL within the herd.The test sensitivity and specificity were held constant at: 0.96 and 0.98 respectively.



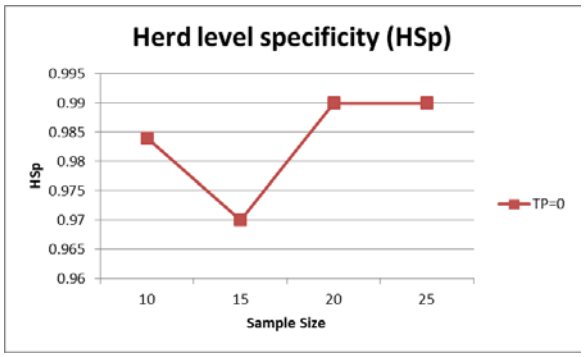


Figure 6.2. Herd-level predictive value positive (HPV+) and herd-level predictive value negative (HPV-) based on different sample sizes (10 to 25) and varying within herd true prevalence (TP; 0.1 to 0.4) of animals with BHBA concentration ≥12 mg/dL and a between herd prevalence (the pre-test probability that a herd is positive) ranging from 0.1 to 0.4.

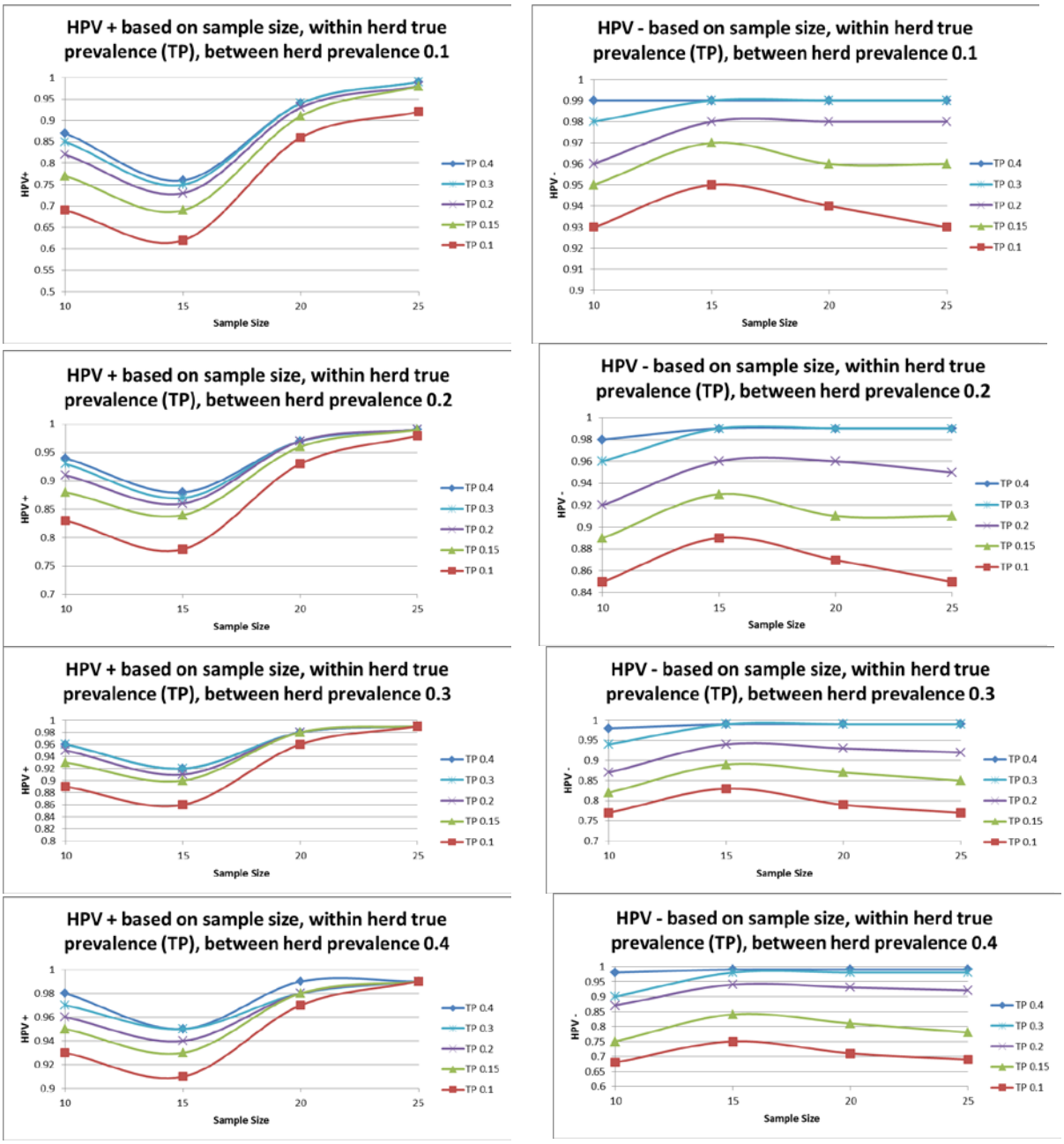
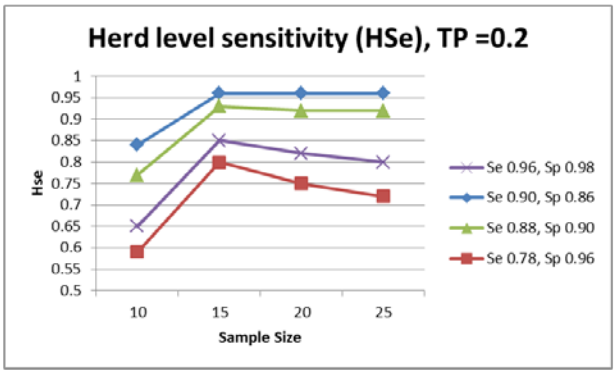
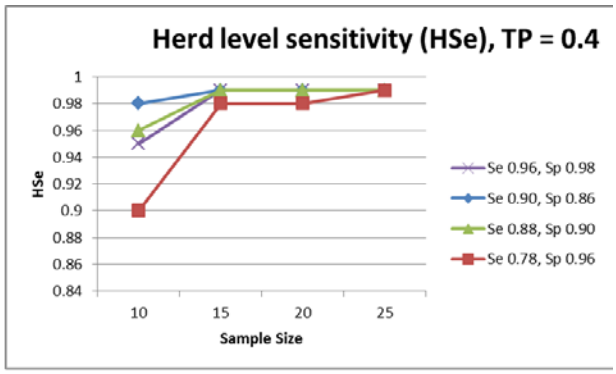
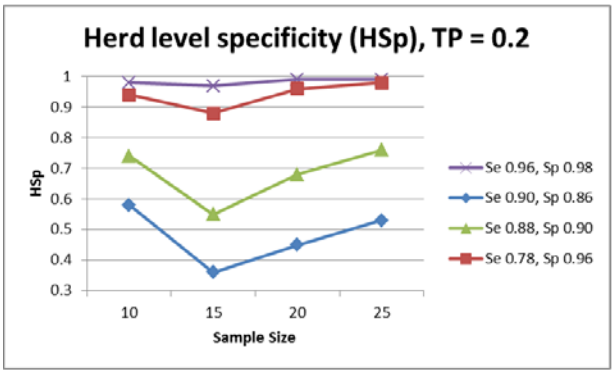


Figure 6.3. Herd-level sensitivity (HSe) and herd-level specificity (HSp) varying test sensitivity, test specificity and underlying true prevalence (TP 20% or 40%) of BHBA ≥ 12 mg/dL.







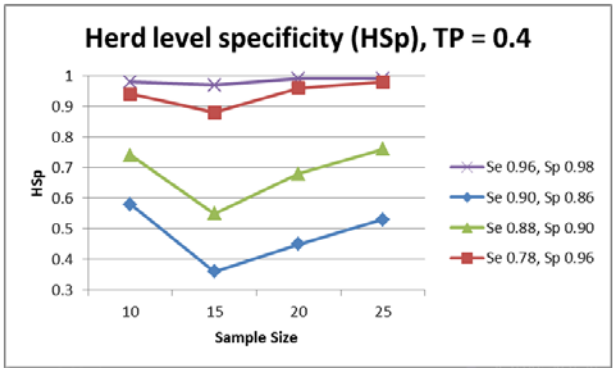
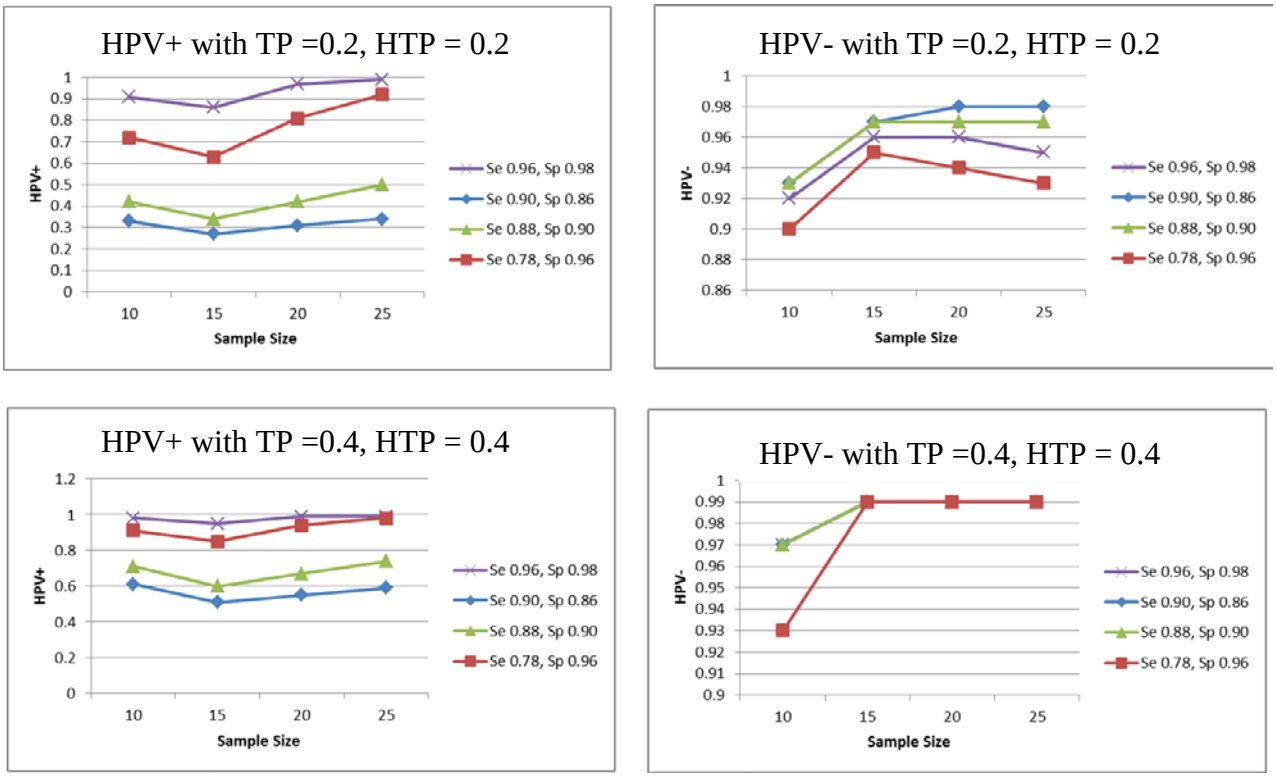


Figure 6.4. Herd predictive value positive (HPV+) and herd predictive value negative (HPV-), comparing true prevalence at 20% and 40% and between herd true prevalence (HTP) at 20% and 40%, varying test sensitivity and test specificity.



DISCUSSION

Pooled samples

A McNemar's p-value < 0.05 can indicate that there is serious bias between the two tests (Dohoo et al., 2003b); however this bias is usually related to subjective interpretation between observers.In the current study, subjective interpretation is not a factor because both tests were based on objective laboratory measures, thus a low McNemar's p-value indicates that the tests were different.

The magnitude of the Kappa value is influenced by the extent of agreement between the two tests, the prevalence of the condition being tested, and bias between subjective observers (Byrt et al., 1993). Despite the differences in the proportion of positive herds between the individual and pooled tests, i.e. prevalence, the Kappa statistic can still be interpreted under the

commonly cited scale (Landis and Koch, 1977).Based on this scale, the tests a Kappa value of 0.13 shows only slight agreement.

The wide confidence intervals for specificity of the pooled test indicate uncertainty about the estimate of specificity and additional herds would need to be tested to improve the accuracy of this estimate.It is plausible that an individual cow, with a very high BHBA concentration could increase the mean of the pooled test above the metabolite threshold, producing a false positive result for excessive NEB.This did not occur in this study because only clinically healthy cows were selected for inclusion in the cohort study and as such, elevated levels of BHBA were rare.

Low test sensitivity can lead to a large number of false negatives which means that herds at risk will not be identified and opportunities for improvement may be lost.These lost opportunities can be very costly because according to Ospina et al., (2010c), herds above the herd alarm level, i.e., when more than 15% of animals tested had BHBA ≥ 12 mg/dL, had a 1.8% increase in DAs and CK, 0.8% reduced pregnancy rate, -358 ME 305 milk (kgs) when compared to herds below the herd alarm level.Therefore, if the goal of testing is to identify herds at risk and capitalize on opportunities, the proportion of animals above the herd alarm level and not the pooled concentration should be evaluated.

Individual tests

Herd level sensitivity and specificity parameters are similar to individual animal parameters, such that a large HSe results in fewer false negatives, and similarly a large HSp results in fewer false positives.In this scenario, the critical threshold is based on a proportion of the animals sampled, the largest HSe is associated with sampling 15 animals, but the highest HSp is associated with sampling a larger sample, 20 to 25 animals.It is at the discretion of the user which outcome is most important.If one is looking for opportunities to improve, the test

with the highest sensitivity would be ideal because false negatives would be limited.However, if the cost of the intervention is prohibitive, a test with higher specificity and fewer false positives may be warranted.In either case, it is important to select clinically healthy animals.Sick animals will likely have elevated markers of NEB.

When reviewing the HSe and HSp, keep in mind that these calculations were based on the individual test Se and Sp and in this study they were maintained at 0.96 and 0.98, respectively. These values are within the range of sensitivity and specificity of the Precision Xtra meter (Abott Laboratories, Abott Park, Il).The Precision Xtra meter is a handheld device used to test blood BHBA concentrations; sensitivity and specificity compared to serum BHBA concentrations determined photometrically are 96% and 98%, respectively, when using a cut-off value of ≥ 1.2 mg/dL (Konkol et al., 2009).There are other ketone tests on the market with varying degrees of sensitivity and specificity.For example: the Ketostix strip (Bayer Corporation, Elkhart, IN) evaluates acetoacetate in urine and when read after 5 seconds and interpreted as a "trace" had 90% sensitivity and 86% specificity and when interpreted as a "small" has a 78% sensitivity and 96% specificity both relative to serum at ≥ 1.4 mg/dL (Carrier et al., 2004); and the Ketotest (Sanwa Kagaku Co. Ltd., Nagoya, Japan) for milk when read at ≥ . .05 mg/dL relative to serum at ≥ 1.4 mg/dL has 88% sensitivity and 90% specificity (Carrier et al., 2004).

Herd-level predictive value tests are the herd-level analogues of the individual tests for predictive values, such that the HPV+ is the probability that a positive tests comes from a positive herd, the HPV- is the probability that a negative tests comes from a negative herd.Given that the critical threshold for calling a herd positive requires that more than 15% of the animals sampled have BHBA concentrations ≥ 12 mg/dL, when 15 animals are sampled this requires ≥ 2 animals to test positive and when 20 animals are sampled, this requires ≥ 3 individual animals to

test positive.By increasing the absolute number of animals required to test positive to call the herd positive, the HPV+ will increase based on sample size.However, because the test used does not have perfect specificity, the more animals tested the lower the HPV- will be due to the increased probability of false positives.

The timing and frequency of sampling are important factors to consider.Although there is some diurnal and feeding time variation of BHBA levels, ultimately the goal is to sample clinical healthy animals at similar time periods within the farm.Sampling at similar times (before or after feeding) will decrease the variation based on this factor.It is important to note that BHBA may be elevated prior to feeding, so in order to increase the sensitivity of the test, one might want to test prior to feeding.

The frequency of sampling depends on herd size and the type of information desired.In herds with fewer than 300 cows, all cows within the risk period (3 to 14 DIM) should be sampled and when the individual test results from a group of 15 to 20 animals have been accumulated, the proportion of positive animals can be evaluated.Based on research done by McArt et al., (2011), it is recommended that animals be sampled between 3 to 10 DIM.In this study, cows were sampled repeatedly up to 17 DIM and the highest incidence of subclinical ketosis (BHBA concentration $\geq$ 12 mg/dL) was seen within 3 to 10 DIM.It is important to note that when the sampling window is decreased to 3 to 10 DIM, the underlying TP of SCK may be higher than in the Ospina et. al., (2010a) study which evaluated animals between 3 to14 DIM.This may result in a necessary increase in the proportion of animals that test positive to determine that a herd is above the herd alarm level.

The frequency of sampling in herds with greater than 300 cows will depend on the information desired.This flexibility is due to the fact that given the number of cows in the herd;

there will be enough cows in the risk period to sample on as needed basis.When evaluating the results at the herd-level, i.e., 15 to 20 samples have already been evaluated, the test can be evaluated in series, where one negative herd-level test will result in the conclusion of a negative overall test.The consequence of testing in series is that the herd-level specificity increases, but the herd-level sensitivity decreases.If the herd-level tests are evaluated in parallel, one single positive herd-level test will result in the conclusion of a positive overall herd-level test.The consequence of testing in parallel is that the herd-level specificity decreases, but the herd-level sensitivity of the test increases.If on-going monitoring is desired, testing in series or parallel every 2 to 4 weeks is recommended.Sampling at these time intervals will allow evaluation of a new population at risk – a new set of healthy animals 3 to 10 DIM.

CONCLUSION

It is normal for dairy cows to have some circulating BHBA; however, there is a threshold above which disease, decreased milk production and decreased reproductive efficiency are more likely.Research about this threshold at the individual animal is useful; however, because management strategies are best implemented at the herd or group level, identifying herds or groups at risk would allow for appropriate intervention. Although pooled samples may seem like a desirable method to evaluate herd-level status due to lower laboratory cost, pooled samples have very low sensitivity when compared to individual samples and result in high levels of false negatives.When relying on representative, individual-animal samples to estimate herd-level status, it is recommended to sample 15 to 20 animals at risk (3 to 10 DIM) and evaluate the proportion of animals with BHBA above the critical threshold.Herds with more than 15% of animals sampled with BHBA concentrations ≥ 12 mg/dL may benefit from evaluation of transition cow management.

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CHAPTER SEVEN

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

The energy management mechanisms in place at the beginning of lactation are beneficial because they allow the cow to produce milk despite having to enter a state of NEB.Based on this system, it is normal for the cow to experience some level of NEB; however, excessive lipolysis, estimated by rising concentrations NEFA and BHBA above critical thresholds are associated with problems during the transition period.The ability for the cow to manage this energy imbalance will dictate whether there are negative effects associated with the NEB, thus resulting in the over-representation of both infectious and metabolic diseases during the early stages of lactation.This research explored the association between NEFA and BHBA, as markers of NEB, and negative downstream outcomes like disease development, decreased milk production, and decreased reproduction.

Association between elevated NEFA and BHBA concentrations and negative downstream outcomes

The first major objective of this research was to identify the critical threshold above which detrimental health outcomes were most likely in individual animals.The health outcomes evaluated were DA, CK, MET, and RP.The use of recently recognized statistical methods with commercially available statistical software for estimation of the risk ratio (**RR**), allowed us to estimate the RR given the dichotomous outcome (disease or no disease) and several covariates,

such as objective concentrations of pre-partum NEFA 0.3 mEq/L, post-partum NEFA 0.7 mEq/L and BHBA 12 mg/dL.

Once the association between critical thresholds of NEFA and BHBA and detrimental health outcomes were established, the relationship between these thresholds and reproduction and milk production were evaluated.In general, this research supported the conclusions of others that excessive NEB was detrimental to reproduction and milk production.However at the individual animal level, primiparous animals behaved differently, milk production was higher in animals with elevated post-partum NEFA and BHBA.Although this warrants further investigation, the difference in response between cows and heifers may be related to additional metabolic demands that a heifer has to balance – skeletal growth and lactation providing an additional demand for the excessive energy mobilized from adipose tissue.

Although these associations at the individual animal level are important, most farm management systems implement changes at the group or herd-level, thus defining an objective herd-alarm level was essential.The herd-alarm-level is defined by two numbers: 1) the proportion of sampled animals with NEFA or BHBA concentrations above the critical concentration threshold, and 2) the critical concentration thresholds that were associated with increased disease incidence and decreased reproductive and milk production performance.

Generally, herds that had more than 15% of the animals tested with pre-partum NEFA > 0.3 mEq/L, post-partum NEFA > 0.7 mEq/L, or BHBA > 12 mg/dL, had higher disease incidence, produced less milk, and had a lower pregnancy rate when compared to herds that had fewer than 15% of the animals with metabolite concentrations above this range.In addition, at the herd-level, heifers did not demonstrate the same association between NEB and milk production

as they did at the individual animal level.The theory is that if enough heifers have elevated NEFA and BHBA concentrations post-partum, there is a negative effect on milk production.

Once information at the herd-level was established, it was also important to evaluate the effect of pooling samples on the interpretation of the herd energy status.Contrary to some of the positive results associated with pooling samples for detection of infectious diseases, pooling samples for evaluation of NEFA and BHBA as predictors of disease, milk production, and reproduction resulted in very low sensitivity.The consequence of low sensitivity is a large number of false negatives, which leads to missed opportunities of addressing energy balance concerns through pooled samples in the transition period.

Directions for Future Research

The association between excessive NEB and negative downstream outcomes was reproduced at multiple levels of this investigation and has been confirmed by other research groups.Although mobilization of fat reserves is the primary mechanism for meeting the demands for energy, objective measures of corporal changes in response to NEB during the transition period are lacking. This is largely due to the fact that access to body weight information is not readily available from most commercial dairy farms.However, farms which have adapted robot technology for milking have an overload of objective information about the weight of the cow because cows are weighed every time they are milked (2-4 times per day).Evaluating the association between NEB through NEFA and BHBA concentrations, objective measures of body weight and back fat thickness (measured through ultrasound) may improve the understanding of the relationship between adipose tissue, body weight, BCS, and NEB.

In addition to evaluating subcutaneous fat, an objective measure of visceral fat may also help improve the understanding of the association between adipose tissue and negative

downstream outcomes.There is some evidence that subcutaneous fat may behave differently than fat that surrounds the visceral organs, and perhaps it is the changes in the visceral fat that may have a stronger association with negative downstream outcomes.The use of ultrasound technology may be used to increase precision and accuracy when evaluating the back fat on cow.Although validation studies would need to be performed, the fat around the kidney can be measured through rectal ultrasound and may help minimize the knowledge gap between what can be seen on the outside and what is going on internally, especially in the cows that do not have excessive body condition subcutaneously.

Lastly, through collaboration with other researchers, two promising areas of research are being investigated.These areas are: the evaluation of decreased metabolite concentrations (when they should be elevated) and the ratio between NEFA and BHBA.Unpublished work on the association between low BHBA concentrations prior to surgical correction of a DA, showed that cows with BHBA concentrations $\leq$ 12 mg/dL were more likely to die or be culled after surgery.This brings up an interesting concept of the exhaustion of energy management mechanisms and warrants further investigation.Following this same logic, the evaluation of the ratio of NEFA to BHBA and the association of this with negative downstream outcomes may improve the understanding of the energy management mechanisms.Although evaluation of this ratio is the next step, my hypothesis for the worst case scenario, i.e., increased risk of negative downstream outcomes, is: high NEFA and low BHBA.This means that ketones are not being produced despite available NEFA, signaling a break down or exhaustion of energy management mechanisms, thus higher risk of hepatic lipidosis and subsequent detrimental immune, reproductive and milk production outcomes.

Final Remarks

Science is the pursuit of the truth.The results presented in this dissertation provide another level which can be used for understanding the real association between energy balance and negative downstream outcomes during the transition period; with the goal of identifying animals or herds at risk and to use this information to minimize the risk of disease, improve milk production and reproduction.Lastly, based on the wisdom of my advisor, Daryl Nydam, who frequently quotes George Box, and says that “although all models are wrong, some models are useful”, I hope the information gained from this research should help advance the understanding of NEB during the transition period.